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EDITORIAL

Endoscopic therapies of gastroesophageal reflux disease

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Abstract

The high prevalence of gastroesophageal reflux disease (GERD) in Western societies has accelerated the need for new modalities of treatment. Currently, medical and surgical therapies are widely accepted among patients and physicians. New potent antisecretory drugs and the development of minimally invasive surgery for the management of GERD are at present the pivotal and largely accepted approaches to treatment. The minimally invasive treatment revolution, however, has stimulated several new endoscopic techniques for GERD.

Up to now, the data is limited and further studies are necessary to compare the advantages and disadvantages of the various endoscopic techniques to medical and laparoscopic management of GERD. New journal articles and abstracts are continuously being published. The Food and Drug Administration has approved 3 modalities, thus gastroenterologists and surgeons are beginning to apply these techniques. Further trials and device refinements will assist clinicians.

This article will present an overview of the various techniques that are currently on study. This review will report the efficacy and durability of various endoscopic therapies for gastroesophageal reflux disease (GERD). The potential for widespread use of these techniques will also be discussed. Articles and abstracts published in English on this topic were retrieved from Pubmed. Due to limited number of studies and remarkable differences between various trials, strict criteria were not used for the pooled data presented, however, an effort was made to avoid bias by including only studies that used off-PPI scoring as baseline and intent to treat.

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Key words: Endoscopic; Treatment; Gastroesophageal reflux disease

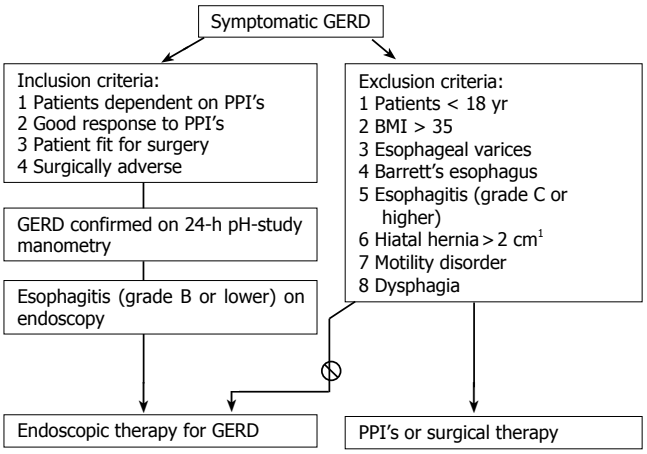
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INTRODUCTION

Gastroesophageal reflux disease affects millions of people worldwide. The prevalence of heartburn in a randomly selected adult population is approximately 20%. It is estimated that approximately one third of the adult population in the United States suffers from heartburn[1]. Of these, approximately 7% have reflux esophagitis. The management of GERD has gained increasing attention during the past two decades due to a high prevalence in Western societies, a better understanding of the pathophysiology, new potent anti-secretory drug therapies, the advent of minimally invasive surgery and new transoral endoscopic therapies. We attempt to determine the true efficacy and benefit of these therapies. The relative advantage of one over the other is enlightened by objective comparison of study outcomes. The potential for use by clinicians in daily practice and the underlying mechanisms of action are also explored.

It is important to understand the pathophysiology of GERD although much controversy remains. The efficacy of the antireflux barrier at the GEJ is dependent upon three factors. These include the "lower esophageal sphincter complex", the geometric profile of the cardia and the changes within each as a result of gastric distension. Other factors such as gravity, intra-peritoneal pressure, esophageal motility and the mucosal barrier also play a role in reflux prevention. Reflux can be precipitated by a decrease in either the length or the pressure of the lower esophageal sphincter (LES) and/or obliteration or diminishment of the angle of His. Both these factors are present during periods of gastric distension. It is believed that transient LES relaxations (tLESR), i.e. intermittent spontaneous decreases in LESP, are responsible for reflux events in patients with a normal LES[2-5]. In the early stages of disease and in the absence of a hiatal hernia, the geometry and integrity of the cardia are normal. However, during periods of gastric distension such as after meals, gastric distension alters the anatomy and makes the sphincter incompetent due to sphincter shortening, which some term transient lower esophageal sphincter shortening (tLESS)[6]. Such patients appear to be the ideal population



[1]Recently some studies have included patients with a hiatal hernias > 3 cm and Barrett's esophagus.

Figure 1 Inclusion/exclusion criteria for endoluminal therapies of GERD.

for endoscopic antireflux procedures.

In the light of the above described pathophysiologic factors, endoscopic therapies should prevent reflux in the following ways; (1) alter the compliance of the cardia and prevent tLES shortening/relaxation, (2) increase baseline LES tone or (3) increase baseline LES length. None of the endoluminal therapies reduce the distal esophagus into the abdomen and effect a hiatal hernia repair.

The majority of patients with GERD are best treated by proton pump inhibitors (PPI's). However, symptom relapse is common after cessation of treatment[7] and lifelong therapy is often necessary. Even while on therapy, up to 33% of patients have recurrent symptoms within the first two years. Almost 50% of patients continue to exhibit objective evidence of acid regurgitation despite complete symptomatic control on PPI therapy. Although, a fundoplication is a good treatment option[8], laparoscopic anti-reflux surgery requires a general anesthetic, hospitalization, postoperative lifestyle limitations for days to weeks, is expensive and is associated with post-operative morbidity and even a mortality rate. In addition, patients may return to medical therapy[9]. These limitations created the need to develop less invasive but effective procedures. These procedures are listed here (1) Endoscopic Suturing devices: Endoluminal Gastroplication (ELGP/Endocinch); Endoluminal full-thickness plicator (NDO plicator); Syntheon ARD Plicator; (2) Radiofrequency energy delivery device: Stretta; (3) Synthetic Implants/Injections: Implantable biopolymer (Enteryx); Implantable Prosthesis (Gatekeeper); Implantable plexiglass microspheres (PMMA). Of these, 3 novel endoscopic therapies have been approved for use by the FDA. All procedures can be safely performed in an outpatient setting utilizing conscious sedation.

The general selection criteria that have been used in most trials are shown in Figure 1. In addition to this, specific patient selection, if any, is mentioned under the respective procedures.

ENDOLUMINAL GASTROPLASTY

Initially, Swain *et al* developed a mechanical aid which allowed passage of a needle and subsequent suture via the biopsy channel of an endoscope[10]. Later, the technique was modified to create plications endoscopically below and at the GEJ for the prevention of GERD.

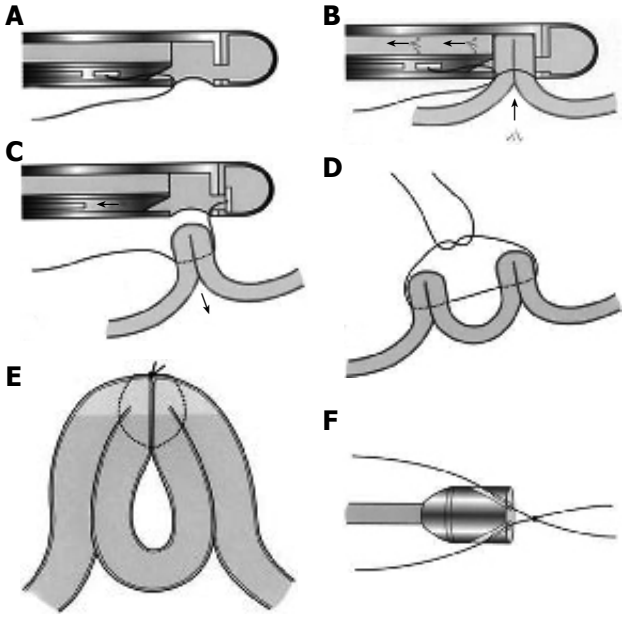


Figure 2 A: Drawing of the suturing device which is equipped with a vacuum chamber and a hollow needle in which there is a suture attached to a tag; **B:** Tissue is drawn into the chamber by suction. The needle passes through the tissue and the tag is captured in the distal chamber; **C:** The suction is then released; **D:** The procedure is repeated on an adjacent piece of tissue; **E:** On tightening the knot, the pieces of tissues are approximated; **F:** The knot, tied outside the animal, is advanced by a knot pusher which is attached to the tip of the endoscope.

Procedure

The procedure requires a suturing capsule, suture tags and an anchoring system that secures the suture and cuts the strands (Figure 2). A short, 18 mm-outer-diameter overtube allows repeated intubations (approximately 12) while avoiding trauma to the esophageal mucosa. Usually patients tolerate the procedure with conscious sedation. However monitored anesthesia or general anesthesia may be used as necessary. Two to four plications are placed either longitudinally (one above the other), radially (next to each other) or spirally within the cardia. Each plication is formed by two stitches that are placed into the gastric submucosa, approximately one centimeter apart and then pulled together. The procedure is completed within 40 to 60 minutes.

The application of cautery on opposing mucosal surfaces prior to plication may secure tissue apposition and promote long-term adherence. Its efficacy has been proved in pilot studies[11] and needs to be tested in a larger randomized controlled trial.

Efficacy

The overall results with ELGP are tabulated in Table 1. This is a pooled data obtained from 11 studies. Two multicenter trials are included in the table and will be highlighted. In the first trial 64 patients were randomized between a circumferential and a linear plication configuration[12]. All patients were dependent on anti-

Table 1 Endotherapy result comparisons: pooled data

Trial result	ELGP	NDO Plicator	Stretta	Enteryx	Gatekeeper[1]
HDQRL improvement	55%	70%	65%	70%	74%[1]
Heartburn improvement	74%	-	61%	71%	-
Off PPI's					
At 1 yr	40%	75%	55%	72%	58%[1]
At > 2 yr	33%	-	63%	65%	-
⩾ 50% reduction in PPI	51%	-	67%	80%	54%[1]
Quality of life improvement					
SF-36 Physical	17%	31%	20%	12%	17%[1]
SF-36 Mental	None	10%	14%	3%	1.4%[1]
Time pH < 4 improvement	16%	None	36%	33%	32%[1]
No. of reflux episode improvement	33%	-	None	31%	45%[1]
pH normalization	25%	-	-	38%	40%[1]
LESP improvement	None	None	None	None	None
LES length improvement	None	None	None	None	None
tLESR improvement	Yes	No	Yes	No	No
Healing of esophagitis	None	None	None	None	None
Sham Trial	3-mo FU	Being planned	1-yr FU	Underway	Underway

[1]6 mo follow-up. None: Change not statistically significant. All results are at 1-yr follow-up except Gatekeeper. Highlighted cell indicate controversy regarding results but the results mentioned above are the ones shown by the majority of studies.

secretories and had proven reflux by 24-h pH monitoring. Manometry and endoscopy was performed to exclude patients with Barrett's esophagus, grade 3 or 4 esophagitis, large hiatal hernias and an esophageal dysmotility disorder. There was no difference found between the plication configuration groups and post-procedure manometry and endoscopy showed no improvement in LES pressure or grade of esophagitis. A significant improvement in heartburn and regurgitation scores from baseline was found but the pH monitoring results although significantly improved showed only a 30% normalization rate.

In a second multicenter study, 85 symptomatic GERD patients when off PPIs and with proven reflux on 24-h pH monitoring were included[13]. Upper endoscopy and manometry were also performed at baseline. Follow up was scheduled at 3, 6, 12, and 24 mo. Symptom scores and medication usage were assessed at each follow up and pH-metry was done at 3 mo follow up. This study was different from others with respect to their inclusion criteria as it included patients with esophagitis grade 3 ($n = 10$), hiatal hernia >2 cm ($n = 9$), Barrett' s esophagus ($n = 4$), failed fundoplication ($n = 3$) and those with pulmonary symptoms ($n = 10$). The majority of the patients had 2 plications performed (range, 1-3) and most had a circumferential plication (65%) with the remaining receiving a linear configuration (35%). Twenty-four-mo follow up data demonstrated durable functional improvement and a sustained reduction in anti-secretory medication. Heartburn scores were reduced at both 1 (94%) and 2 yr (78%) follow up. At 2 yr follow up, complete resolution for heartburn was seen in 52% of patients and 77% for patients with regurgitation. Likewise, PPI usage decreased with 69% of patients using <50% of their baseline medication and 41% were completely off PPI's at 2 yr follow up. The duration and number of esophageal acid exposures at 3-6 mo post-procedure were significantly reduced. There was no change in LES length or pressure when measured at 3 mo.

A sham-controlled, randomized, blinded single institution study ($n = 34$) is underway and 3 mo follow-up is available[14]. The study nurse and patients were blinded to the procedure performed. An overtube and 2 endoscopes were exchanged in all patients and the conscious sedation dosing was similar. Four circumferential plications were placed when performing the ELGP. At 3 mo follow up, heartburn frequency (69% *vs* 31%, $P = 0.03$) and severity (47% *vs* 17%, $P =$ NS) were improved in patients with ELGP in comparison to those with the sham procedure. A significantly greater number of patients discontinued their daily PPI/H_2B (75% *vs* 25%, $P = 0.03$) in the plication group. A significant reduction in % time pH < 4 was also observed, however, no difference was seen between groups regarding normalization of pH, median LESP or quality of life. Limitations of this study include a probable type II error, a larger than expected sham effect, inadequate follow-up length and lack of technique standardization. A randomized controlled trial of larger size with longer follow up is needed for objective evidence of durable benefit.

Other studies of note have demonstrated a markedly improved quality of life at one year follow up[15], reduction of the rate of tLESRs by 37% at 6 mo in a single-center study[16], in baboons[17] a significant increase in LES length, and Kadirkamanathan *et al* reported an increase in intra-abdominal, but not total, length of the LES after placement of three linear plications in dogs[10]. A double blind pilot study with 18 patients randomized to either ELGP alone or ELGP with cautery (10 in cautery group, 8 in no cautery group) showed numerically improved patient plication persistence, decreased esophageal exposure and improved symptoms at 1 year in the cautery group. However, the benefits were not sustained at 2-yr follow

Table 2 Procedure characteristics and complications: Pooled data

Variable		Endocinch	NDO Plicator	Stretta	Enteryx	Gatekeeper
Procedure duration (mean)		68 min	20 min	69 min	33 min	35-60 min
Personnel required		1 physician and 2 assistants	1 physician and 2 assistants	1 physician and 2 assistants	1 physician and 2 assistants	1 physician and 2-3 assistants
Sedation required		Conscious sedation 82%	NA	Conscious sedation 100%	Conscious sedation 100%	NA
Approximate no. of procedures performed		4000	NA	4000	2600	225
Major complication	Perforation	0.08%	0.00%	0.13%	0%	0.40%
	Bleeding	0.05%	NA	0.05%	0%	0%
	Hypoxemia	0.08%	0%	0%	0%	0%
	Pleural effusion	0%	0%	0.03%	0.08%	0%
	Pericardial effusion	0%	0%	0%	0.08%	0%
	Aspiration pneumonia	0%	0%	0.05%	0%	0%
	Esophageal abscess	0%	0%	0%	0.04%	0%
	Ulceration over prostheses	0%	0%	0%	0%	0.40%
	Death	0%	0%	0.05%	0.04%	0%
Minor complications		• Sore throat (0.35%) • Chest soreness (0.17%) • Abdominal pain • (0.15%) • Bloating (0.02%) • Transient dysphagia (0.05%) • Bronchospasm (0.01%)	-	• Superficial mucosal injury • Burn at pad site (0.02%) • Transient A. fib (0.02%) • Bloating (0.02%) • Gastroparesis and ulcerative esophagitis (0.02%) • Low grade fever • Transient dysphagia • Transient chest pain • Topical anesthesia-related complications (eg. Allergy, hypotension)	• Garlic odor for several hours (due to DMSO) • Chest pain (82%) • Transient dysphagia (13%) • Belching/ burping • Bloating/ flatulence • Fever	• Sore throat (15%) • Chest pain (5%) • Nausea/ vomiting (0.8%) • Erosive duodenitis (0.8%) • Retrosternal pain (0.4%) • Poor sleep (0.4%) • Abdominal pain (0.4%) • Rash (0.4%) • Cough (0.4%)

NA: not available.

up[11]. Plication persistence at 1 year was 37% in the cautery group *vs* 15% in the no-cautery group. Further studies are required to elucidate the efficacy of cautery with ELGP.

Changes in selection criteria

A significant improvement in symptom scores and pH study results has been observed in 19 patients refractory to medications, although, this improvement was less than that seen in other studies[18]. Short term studies suggest that ELGP can be used as an effective salvage procedure for failed surgical fundoplication[19,20], however, this indication requires further study.

Mechanism of action

The mechanism by which ELGP improves competence of the gastroesophageal junction remains unclear. Feitoza *et al* demonstrated lack of fusion between the folds when sutures were placed intra-luminally in the stomach of rabbits, irrespective of suture depth[21]. The symptomatic improvement can be explained by a lower volume of refluxate reaching the more "sensitive" proximal esophagus. The decreased volume might signify a decreased frequency of tLESR's, probably due to scar formation. Secondary scarring may also impair distensibility of the proximal stomach. This when combined with the fact that ELGP has been shown to stimulate localized circular muscle hypertrophy in both humans and animals[22] may lead to an increased basal tensile tone and increased resistance to gastric distension. This explanation does not negate, however, the deleterious effect of the acid within the distal esophagus and the chance for progression to complications such as stricture formation or even Barrett's esophagus.

Complications

Endoluminal gastroplasty is generally safe over long term follow up and free of serious immediate side effects. The major and minor complications are shown in Table 2. Esophageal perforations can occur. Two perforations have been seen so far, one of whom required a thoracotomy and the other hospitalization and antibiotics. To avoid a perforation, which is usually due to placement of a full-thickness suture within the esophageal wall, all stitches and plications should be placed below the squamocolumnar junction. If the suturing capsule needle does not retract after penetrating the tissue, the handle with pusher rod should be disassembled rather than pulling the capsule away from the esophageal wall. Occasionally, the suture loops and locks at the tissue level as the second stitch is being placed. The needle literally goes through a loop and the suture will not slide through the tissue on removal of the endoscope. In this circumstance the suture should be cut with an endoscopic scissors, however, this can be difficult and pull out may be necessary. If tissue accompanies the knot, it should be sent to pathology for frozen section analysis. If the muscularis propria is

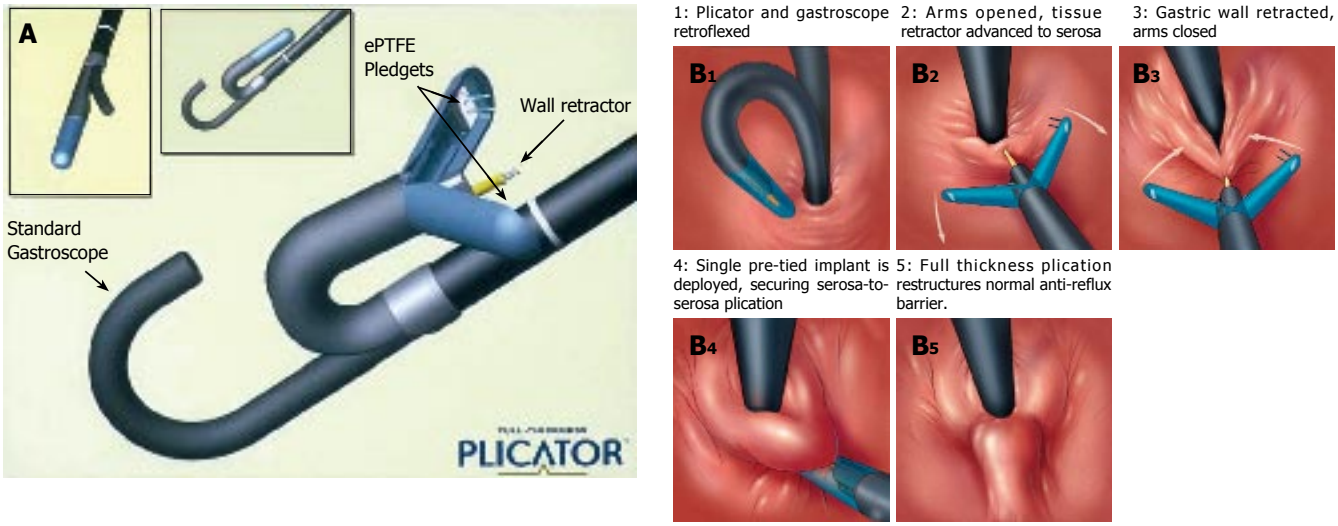


Figure 3 A: The NDO plicator mounted on a small diameter endoscope; **B:** Schematic representation of the procedure for application of a full-thickness plication by NDO plicator.

included within the specimen, an esophagram should be performed followed by hospitalization.

Procedure failure

Studies demonstrate that laparoscopic Nissen fundoplication (LNF) is feasible and effective after failed ELGP[23,24]. Patients should undergo upper GI endoscopy before surgery but suture removal is not necessary. No significant scarring or adhesions have been noted in the esophageal hiatus or inferior mediastinum at LNF. This may be due to the stitches not penetrating beyond the muscularis propria, as penetration of the serosa has been shown to induce more scarring[21]. This in itself might contribute to the lack of durability in post-ELGP results. Although the technique is initially effective, long-term symptom control has yet to be established.

Alternatively, patients who experience recurrent GERD symptoms post-ELGP may benefit from a second procedure. However, one study demonstrates a significant trend toward earlier onset of recurrent symptoms after repeat ELGP[25].

Advantages

Easy repeatability, short operative time, early discharge, no morbidity and symptomatic improvement make ELGP an attractive option. Endoscopic gastroplication has proven short term efficacy and has been demonstrated to be cost-effective for one to two years[26].

Disadvantages

The absence of objective improvement after ELGP is disconcerting. No studies have shown improvement in LES pressure or length and grade of esophagitis. pH monitoring results are mixed but the rate of normalization is only 30%-40% between studies. Several studies have compared ELGP with LNF[27-29]. Comparable improvement in symptom scores, reduction in PPI intake and QOL assessments has been seen, however, surgery is superior to ELGP in patient satisfaction and objective improvement in reflux.

Recently, Chang *et al* showed that ELGP with cautery did not improve LESP, Gastric yield volumes, pressures or GEJ compliance in a porcine model, however, limited sample size ($n = 5$) precludes definitive analysis[30]. Lack of durability is another reason for concern as shown by many authors. Lepoutre *et al* reported loss of 51% plications in 60 patients at one year follow up[31].

The optimal configuration for plications is not known. In a small and unfortunately underpowered study by Davis *et al* 22 patients with proven GERD were randomized between a helical and circumferential plication pattern. No difference in outcome was observed between configurations at 18 mo although a trend in objective results favored the helical pattern[32]. At 18-mo follow up, only 15% of patients being asymptomatic and off antisecretory medication. The prevalence and persistence of these possible advantages are currently subject to investigation. Increasing the number of plications, when using the helical pattern, did not show a significant benefit at either 6- or 12- mo follow-up[32].

ENDOSCOPIC FULL-THICKNESS PLICATOR

Current endoscopic suturing techniques usually involve submucosal suture placement, which may limit potential procedure-related complications but may also lead to early suture dehiscence and loss of long term efficacy. This problem may be theoretically solved by full thickness suturing or stapling devices, which include pledgets. Such an approach may, however, increase the risk of subsequent perforation. A novel technique of applying a full-thickness plication endoscopically has been developed and recently underwent clinical study in a U.S. multicenter trial. Selection criteria were similar to that shown in Figure 1.

Procedure

The (NDO) plicator (Figure 3A) is designed to apply a full thickness pledget reinforced U stitch tissue near the

GE junction with serosa to serosa apposition. The system consists of a reusable instrument and a single-use suture-based implant. Additionally, a proprietary endoscopic tissue retractor and a 5 mm diameter endoscope are used to perform the procedure[33]. The tissue retractor is designed to engage the deep gastric wall, allowing for creation of the serosa to serosa plication.

With the patient under conscious sedation, a gastroscope is passed into the stomach for endoscopic inspection and passage of a guidewire. The gastroscope fitted with the plication device is passed into the stomach over the guidewire. The suture applicator is retroflexed and properly positioned under endoscopic vision. The endoscopic tissue retractor is then inserted to within 1 cm of the GE junction and advanced to the level of the serosa which is judged by visible tenting of the tissues around the entry point of the retractor. The gastric wall is then retracted and instrument arms are closed upon it. The pre-tied implant is deployed to secure a full-thickness plication. The tissue retractor is removed from the greater curvature side of the GEJ, the jaws opened and entire assembly is removed from the stomach followed by removal of the overtube (Figure 3B 1-5). The approximate procedural time is 15-20 min.

Efficacy

Pooled results for NDO plicator are shown in Table 1. A multi-center study enrolled 64 patients with symptomatic GERD who were dependent on antisecretory medication and showed evidence of esophageal acid exposure (pH-metry) without an underlying motility disorder. Follow up was completed at 1, 3 and 6 mo post-procedure. It showed a significant reduction in GERD symptom scores, medication usage and esophageal acid exposure on 24-h pH-study, which persisted at one year follow up. No significant changes in esophageal manometry were noted[34]. At 6-mo follow up, there was a 63% symptom score improvement with elimination of PPI therapy in 74% and normalization of pH in 30% patients. No patient required re-treatment during the 6-mo follow up. In this initial trial, one full thickness plication was used. A pilot study on 7 patients also showed similar results but failed to show objective improvement of reflux[35]. Further studies with single *vs* multiple plications are anticipated. No sham controlled trial has yet been completed but one is being organized.

Mechanism of action

This procedure aims at inducing fusion of the opposed gastric tissue and thereby reducing distensibility at the GEJ. Feitoza *et al*[21] showed maximal fibrosis with incorporation of serosa in the plication when compared to other depths of suture plication. Theoretically, the suture retention rate and thus, durability of results should also improve. Lengthening of the intra-abdominal segment of the LES[33] is expected.

Complications

The most common complication was sore throat (spontaneously resolving within several days post-procedure).



Figure 4 Syntheon device.

One gastric perforation did occur during the multi-center trial and was managed conservatively.

SYNTHEON ARD PLICATOR

This is a promising new endoluminal suturing/compression implant device that has the capacity to place two titanium plications at once, thus, decreasing technical variability between operators (Figure 4). The distance between the two stitches is pre-determined. The plications are removable within the first 48 h. Withdrawal of the device is not necessary after each stitch which may decrease the procedure time and anesthesia related complications. Implantation is performed using a standard endoscope and no overtube is required. The mean procedure time is 21 min. Initial results show the device to be safe and time-efficient. An expanded multicenter pivotal phase I study is ongoing and will help clarify the procedure results.

Efficacy

The results of feasibility trials involving 8 patients with symptomatic GERD, abnormal pH scores, normal motility and responsiveness to daily PPI use have been published. Patients with large hiatal hernias (>2 cm), esophagitis (grade II or more) and Barretts esophagus were excluded. At 6 mo follow up, 75% were off PPI's, 68% improved GERD-HRQL score and 40% improved GSRS:GERD score. However, 24-h pH and manometry showed no significant improvement. (S1199)

Complications

Serious complications have not been encountered. Minor adverse effects seen to date are sore throat, epigastric/referred chest pain and the gas bloat syndrome.

THE STRETTA PROCEDURE

Radiofrequency energy (RFe) has been extensively utilized in medicine since 1921 and is currently being used in the treatment of benign prostatic hypertrophy, cardiac arrythmias and metastatic liver lesions. The possibility of RFe being used for GERD therapy was explored after successful treatment of snoring and sleep apnea.

Patient selection

Radiofrequency augmentation of the LES has been widely used. Over 3500 procedures have been performed in the United States alone. The indications in the past have

been confined to patients with early reflux disease. The Stretta procedure may have specific utility in morbidly obese patients, those with a previous gastric resection, a gastric bypass, after a failed LNF[36] or as an alternative to re-operation after fundoplication disruption. In a porcine fundoplication disruption model, fluoroscopic guidance improved RF lesion accuracy therefore it has been suggested that fluoroscopic guidance be utilized to ensure probe placement[37]. Patients with failed anti-reflux surgery and subsequent RF therapy have experienced non-significant symptomatic improvement (heartburn score from 3.33 pre-procedure to 2.75 post-procedure), however, patient satisfaction scores are significantly improved[38]. The role of the Stretta procedure in postoperative fundoplication patients remains unclear.

Procedure

The Stretta catheter (Figure 5A) is a flexible, handheld, disposable 20F Savary-style dilator that is used in conjunction with the Curon (Sunnyvale, California) control module. It is comprised of a balloon basket assembly, 4 nickel titanium electrodes, suction and irrigation. The balloon basket deploys 4 radially arranged electrodes into the smooth muscle of the GE junction. Tiny thermocouple temperature sensors within the electrodes provide temperature feedback to the radiofrequency generator. A target temperature is pre-selected and the power is automatically discontinued if the temperature exceeds the predetermined threshold. The needles also provide feedback on impedance which allows the operator to know if the needles are positioned correctly into the tissue.

Under conscious sedation, the catheter is placed over the guidewire into the stomach and withdrawn to the position of needle deployment. After removal of the guidewire, the balloon at the distal end of the catheter is inflated, the electrodes are deployed and RF is applied for a specific period of time while monitoring the temperature and impedance levels (Figure 5B). A first treatment ring of 8 lesions is created by rotating the catheter 45° and repeating the same. Four such antegrade rings, each with 8 lesions, are created at 0.5 cm intervals to a distance of 1cm below the GE junction. Two further gastric "pull-back" rings, of 12 lesions each (3 sets of deployment each), complete a set of thermal lesions (Figure 5C). Halfway through the procedure, an endoscope is reintroduced to verify the location of treatments with subsequent adjustment distally or proximally to prevent superimposition of lesions. Following recovery, patients continue their usual anti-reflux medication for 3 wk. A modified technique has been proposed in the presence of a large hiatal hernia (>3 cm) or a failed Nissen fundoplication[39]. The modified technique creates 6 anterograde treatment levels instead of 4, beginning 1 cm above the squamocolumnar junction with 5mm between levels and placing 2 sets of lesions at each of the 4 proximal levels and 3 sets at the distal 2 levels.

Efficacy

In the initial US open label trial[40], one year follow up showed a significant decrease in symptom scores. Gastro-esophageal reflux disease-specific quality of life satisfaction scores and distal acid exposure were significantly improved over the baseline on-medication scores as well. However, normalization of pH monitoring scores did not occur in the majority of patients. In addition, LES pressures did not increase and esophagitis did not improve significantly at 6 mo endoscopic follow-up.

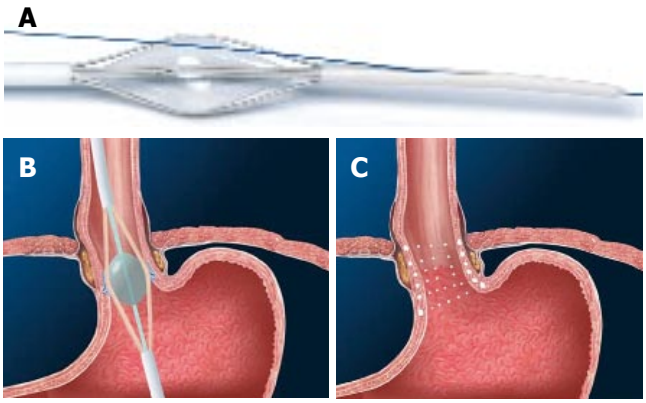


Figure 5 A: The Stretta catheter with guidewire; **B:** The balloon assembly with struts and electrodes extending into the muscularis propria; **C:** A total of 56 lesions are applied as seen in this diagram.

The sham-controlled trial[41] showed significant improvement in symptom scores and quality of life at 6 and 12 mo, but at 6 mo there was no difference between groups in medication usage. Esophagitis grade did not show improvement, and in fact grade 2 esophagitis increased in severity in both groups. Similarly, pH scores also failed to show significant improvement, unlike the previous uncontrolled studies[40,42]. The explanation for these findings may be altered sensitivity of the distal esophagus or unusual persistence of the sham effect.

In a registry series[43], 558 patients underwent the Stretta procedure and showed a significant improvement in GERD symptom control (from 50% to 90%) and patient satisfaction (from 23% to 86% at a mean follow up of 8 mo. The onset of GERD relief was in less than 2 mo in most patients (69%). The treatment effect was durable beyond 1 year and most patients (51% at one year *vs* 96% pre-procedure) were off all antisecretory drugs at follow-up. Most studies are limited to short term follow up (up to 12 mo). For the first time, Torquati *et al* reported long term results in 41 patients with 83% being highly satisfied at a mean follow up of 27 mo[44]. Proton pump inhibitor usage was discontinued in 56% patients and was significantly reduced in 87%. Similarly, Reymunde *et al* demonstrated significant and sustained improvement in anti-secretory drug discontinuation (88%), GERD symptom scores (82%) and QOL scores (44%) at more than 3 years of follow-up[45]. This is encouraging evidence and suggests that durability of results is the distinguishing attribute for the Stretta procedure.

Significant improvement in symptom scores and quality of life have also been observed in shorter term studies[40,43,46,47]. Similarly, decreased PPI use and increased patient satisfaction have been consistently seen[46,40,47]. Most studies fail to demonstrate a beneficial effect of Stretta

on esophagitis but Triadofilopoulos *et al*[42] showed an improvement in esophagitis grade, from 21% with grade 2 esophagitis to 9.3% post-procedure in 43 patients at 6 mo. The role of Stretta in improving extra-esophageal manifestations of GERD is still not clear, however, one study[36] suggests that respiratory benefit can be achieved if strict patient selection (abnormal pH study) is followed. Pooled results are shown in Table 1.

Mechanism of action

Multiple changes may be responsible for the effect seen in the Stretta procedure. First, a mechanical alteration and thickening of the LES musculature leads to diminished reflux, as shown in the canine model[48]. Secondly, progressive tissue remodeling and scar formation observed after Stretta could contribute to the decreased compliance and an increased tensile strength of the GE junction which also exerts its effect in decreasing tLESRs. This decrease in tLESRs has been shown in both animals and humans[48,49]. Histologically normal muscle with occasional focal areas of collagen deposition at 8 wk follow up has been shown in the porcine model.

The disparity between symptomatic improvement and acid exposure seen in the sham trial was surprising and might be explained by a residual sham effect on symptoms or altered visceral sensitivity[41]. The later would explain the striking dichotomy between symptom relief and minimal to moderate improvement in acid reflux profiles. A diminished sensitivity may be due to destruction of chemosensitive or mechanosensitive nerve endings[50]. However, some studies contradict this contention[44] by showing significant improvement in pH results at 27 mo in patients who had responded to therapy.

Complications

The major and minor complications are shown in Table 2. The 5 perforations and 2 deaths occurred during the learning curve and were due to poor patient selection and technical errors. No major complications were seen in the multi-center trial and subsequent studies have not shown adverse effects on vagal nerve function or gastric emptying. The complication rate after Stretta has been $<0.6\%$ since the introduction of the new technology and 0.13% in the last 12 mo.

Recommended precautions

Operators should check the position if abnormal impedance/temperatures are observed, use correct balloon pressures, control the mucosal temperature carefully, minimize balloon pull back pressure and avoid NG tube placement for one month post-procedure

Alternatives after treatment failure

In the US open label trial, 5% of the patients elected to undergo fundoplication 6-12 mo after Stretta due to incomplete or recurrent symptom response. In each case, there was no evidence of extra-esophageal tissue abnormality and the anti-reflux operation was performed in a normal manner and without difficulty. Richards *et al*[36] compared the outcome of patients who had the Stretta procedure and a laparoscopic fundoplication at 6 mo and found a comparable and significant improvement in QOLRAD, SF-12 and pH scores in both groups. However, medication usage was significantly less in patients who had surgery (97% *vs* 58% off PPI's). Both groups were highly satisfied with their procedure. A repeat Stretta procedure is not recommended.

ENTERYX

Enteryx consists of a biocompatible polymer (80 g/L EVOH with a radioopaque contrast agent dissolved in an organic liquid carrier [DMSO]). Upon contact with tissues or body fluids after injection, the solvent, DMSO, rapidly diffuses resulting in precipitation of the polymer (EVOH) as a spongy mass. It is not biodegradable and has no antigenic properties. Migration through blood vessels or lymphatics nor prosthetic contraction after injection have been observed[51].

The three components of Enteryx, ethylene vinyl alcohol polymer (EVOH), dimethyl sulfoxide DMSO, and tantalum, have been previously used together medically as a vascular embolization agent and a membrane for hemodialysis and plasmapheresis.

Patient selection

Enteryx is a treatment with promise but, like other endoluminal therapies, is lacking objective supportive data. Patients must understand that the procedure is irreversible[52]. The possible future indications for Enteryx include primary therapy for GERD in patients who respond to PPI's but prefer not to take medications daily, salvage therapy for PPI responders to reduce or eliminate daily medications and salvage therapy for surgical failures[52]. Recently, Enteryx has been attempted in pos-fundoplication patients[53].

Enteryx therapy is clearly contraindicated in any individual who does not have physiologically documented GERD by pH study or endoscopic findings. It is also contraindicated in individuals who cannot undergo or tolerate endoscopy and those who have esophageal varices. There is no reported experience with this procedure in individuals with esophageal motility disorders, prior gastric or GERD surgery, scleroderma, Barrett's esophagus, hiatal hernias >3cm, BMI >35 or patients who use anticoagulants other than aspirin[52].

Procedure

The procedure is performed in an endoscopy suite equipped with fluoroscopy under conscious sedation. A long needle catheter is filled with Enteryx after it has been flushed with DMSO, to keep it liquefied as long as it stays within the catheter. The prepared injection needle is placed into the muscularis propria at the appropriate level of the esophageal wall. The prosthesis is injected at a rate no greater than 1mL/s under combined fluoroscopic and endoscopic guidance (Figure 6). The EVOH solidifies within the esophageal wall as the generated heat causes DMSO to dissipate. The injection is stopped if either a submucosal or transmural injection is observed. Submucosal accumulation of material is seen endoscopically as a black bulge and an extramural

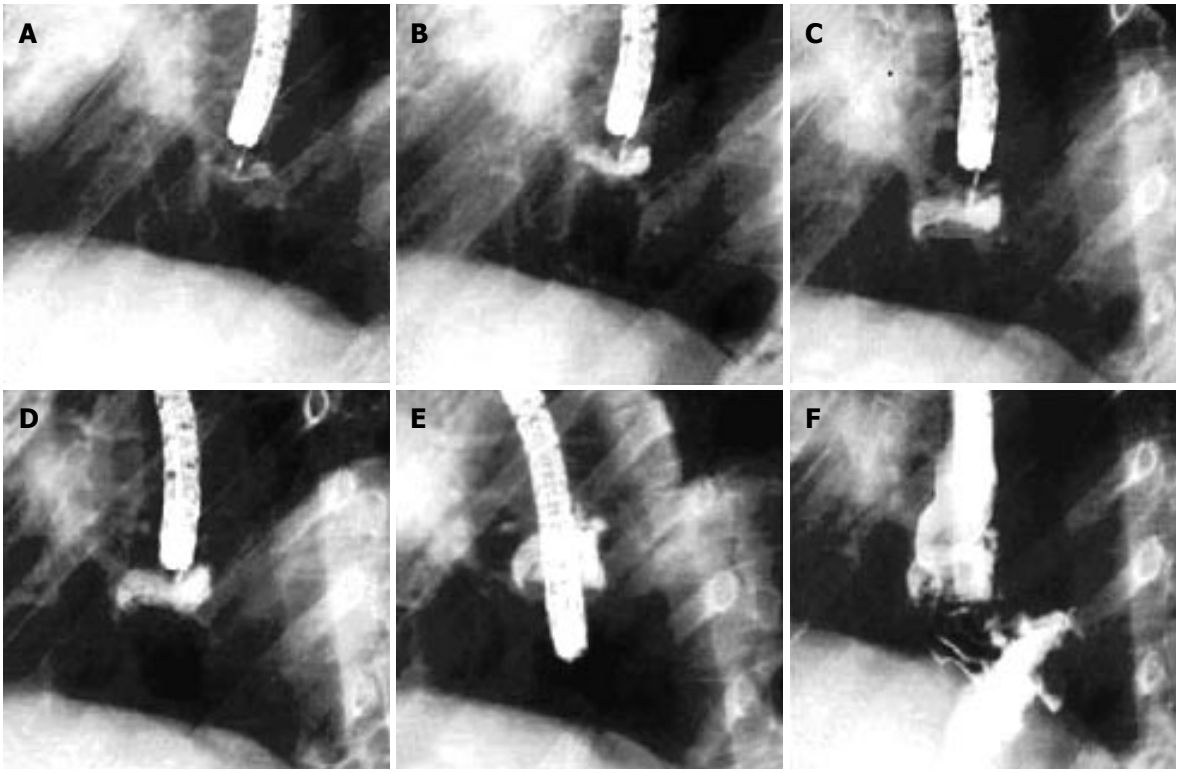


Figure 6 Enteryx injection under fluoroscopic control with a resultant prosthetic cuff.

injection is demonstrated on fluoroscopy as either flow of material beyond the muscularis into the mediastinum or the abdominal cavity, or lack of a visible deposit in the esophageal wall. If a circumlinear transverse path of material is visualized under florosocopy, the injection is completed at this stie using a total of 1 to 2 mL. The procedure is considered satisfactory if 6 to 8 mL of Enteryx is delivered to the muscularis propria circumferentially without a submucosal or transmural injection. After the injection is complete at one site, the needle remains in place for 20 s allowing the material to stabilize and solidify. This maneuver prevents leakage of the prosthesis into the esophageal lumen. Patients are usually discharged 2 to 4 h after recovery.

Robert *et al* reported Enteryx implantation in 5 pigs without the use of fluoroscopy. Enteryx was consistently deposited into the deep esophageal wall with a high degree of accuracy in a minimal amount of time. The placement was accurate in 85% and was transmural in just one instance. A human trial is underway to confirm these findings[54].

Efficacy

Enteryx implantation significantly improves the QOL scores and medication usage[51,52]. No change has been seen in the severity of esophagitis at endoscopy after Enteryx implantation. In general, the structural characteristics of the LES, including its length and pressure, are not altered significantly. Pooled results are shown in Table 1.

The 2 year follow up results of this U.S multicenter trial, which included 85 patients, were recently published[55] and showed PPI use to be eliminated in 74% of patients at 6 mo follow up. This effect was maintained in 64% of subjects at 2 years while 74% were maintained on less than half of their baseline PPI dosage. The improvement in symptom scores was 82% at 6 mo and 70% at 2-yr. Quality of life (SF-36) questionnaires demonstrated an improvement of 6% from baseline at 3 mo and 3% at 12 mo for the mental score while the improvement in the physical score was maintained at 12% at both 6 and 12 mo follow up. pH scores improved with 30% normalization and there was a small but significant LES length augmentation (1 cm) following therapy[56]. No significant change in LES pressure was observed. The absence of change in LES resting pressure contrasts with findings from the pilot study, in which a significant increase in the LES pressure was observed at 6 mo. This may have been due to the inclusion of patients with a normal LES pressure at baseline or the smaller sample size in the pilot study. Importantly, most of the decline in treatment responders during follow-up occurred between 1 and 6 mo. Between 6 and 12 mo, the proportion of treatment responders remained stable. There was no evidence that the reduction in PPI usage after the implant procedure was due to medication shifting.

The decline in residual implant volume seen after one month was attributable to sloughing of superficially implanted material until encapsulation was complete. There was no radiographic evidence of implant migration and after 3 mo, residual volume remained stable ($P > 0.1$)[57]. Twenty two percent of patients were re-treated at 3 mo follow up of which, 63% improved at 12 mo follow up with 58% completely off PPI's and 5% of patients reducing their PPI usage by more than 50% of baseline[56].

The multicenter randomized controlled sham trials with "cross-over" option starting at 3 mo post-randomization are currently underway in both Europe and the United States[58]. An interim report on 56 out of the 64 total

European patients has been announced with 3 mo follow up. An improvement in GERD HQRL was seen in 65% patients in the Enteryx arm *vs* 21% in the sham arm. The median change in symptom scores was 15 for the Enteryx group and 4 for the sham group. There was also a greater reduction in PPI usage (64% *vs* 33%) and a lower cross-over/re-treatment incidence (21% *vs* 71%) in the Enteryx group when compared to the sham group. Criteria for both cross over (for controls) and re-treatment (for the Enteryx group) were the same i.e. an off-PPI HQRL score >15[58].

Johnson *et al* reported that the likelihood of a successful clinical outcome is higher with more residual implant volume. He showed that all patients who retained $\geqslant 5$ mL of implant material eliminated or reduced PPI use by $\geqslant 50\%$ and the majority of subjects who retained >5 mL of Enteryx achieved a GERD-HRQL score of <15. Lehman *et al* reported the procedure to be equally effective irrespective of the radiologic pattern evident at the time of implantation[59]. In a study evaluating predictors of outcome for Enteryx, Deviere *et al*[60] showed there was no statistically significant difference in PPI usage or pH outcome by gender, but GERD-HRQL symptom scores were significantly more likely to improve in males (86%; 57/66) than in females (67%; 32/48) ($P=0.01$). Finally, Ganz *et al* compared the endoscopic findings of patients from the multicenter study at 1 yr follow up with their baseline values (on PPI's) and reported that treatment with Enteryx provided improvement in esophagitis scores comparable to that provided by PPI medication[61]. This finding, however, has not been supported by other studies. A pilot study determining the efficacy of Enteryx in post-fundoplication patients ($n=19$) and reported no apparent difficulty due to the distorted anatomy during the procedure. Post-fundoplication patients with refractory symptoms on high dose PPI therapy were enrolled and 'improvement' was seen in 89% of patients, however, this study primarily addressed the technical feasibility rather than the efficacy of post-fundoplication Enteryx implantation[53].

Mechanism of action

Both gross and histological examination in animals have shown that the implants persist as encapsulated, firm, smooth, slightly mobile ovoid masses, several weeks after implantation. No evidence of pathologic inflammatory changes in the surrounding tissues have been observed. However, the implants lead to a significant increase in the yield pressure and yield volume with a raised threshold for transient relaxations after implantation[6].

Fibrous encapsulation may functionally lengthen the LES. The encapsulation/scarring is the probable mechanism of effect but the exact mechanism in humans remains to be fully characterized. The "bulking" effect, seen with some injectable treatments for urinary incontinence, is not apparent for Enteryx because follow-up endoscopies reveal no evidence of luminal narrowing. Sloughing of superficially injected material may be the reason for inadequate clinical outcome and lack of durability seen in some patients[57].

Complications

The complications are shown in Table 2. Recently, one death was reported in a patient due to inadvertent injection of Enteryx into the aorta. Further details are not yet available. Two patients experienced a pericardial effusion after injection of the prosthesis and subsequently underwent a pericardial window. Two additional patients developed a pleural effusion but no other problems were recognized. Although, the cause of the effusions is not clear, extension of an inflammatory process from the esophagus to the surrounding structures namely pericardium and pleura and the possibility of an allergic or infectious process cannot be ruled out.

Recommended precautions

All operators are required to receive hands on laboratory training prior to clinical utilization. Injection techniques when using fluoroscopy are emphasized and guidelines for prostheses preparation are given. Most centers place patients on a liquid diet followed by a soft diet, the day of the procedure followed by normal diet the day after. Maintenance therapy with PPI's is continued for 10-14 d after implantation.

GATEKEEPER

Gatekeeper is a dehydrated hydrogel prosthesis implanted into the submucosa of the cardia/LES. It hydrates to 6 mm × 15 mm cylinder shaped soft pliable cushions and is removable by endoscopy[62].

Patient selection

In addition to the general selection criteria already described, patients with esophageal varices, a peptic stricture and morbid obesity were excluded from the trials for Gatekeeper.

Procedure

The Gatekeeper device consists of an overtube with separate channels for passage of an endoscope and a long delivery sheath (Figure 7). After placement of the overtube, the injection capsule is placed through the overtube to straddle the squamocolumnar junction. A vacuum is created to stabilize the device and to draw the mucosa in. The injection needle is advanced into the submucosa followed by injection of 3-6 mL of sterile saline until blanching is observed. This is followed by removal of the injection needle and advancement of the needle assembly and the delivery sheath into the mucosa, leaving the delivery sheath in the submucosal plane. After the needle assembly has been retracted, the prosthesis is inserted into the proximal end of the delivery sheath and advanced to the submucosal level using a pushrod assembly. Up to 6 hydrogel implants are placed. The implants are small and "sliver"-like when introduced but swell to full size within 24 h, when hydrated.

Efficacy

In a limited number of patients the Gatekeeper procedure has been shown to significantly decrease heartburn,

improve quality of life, 24-h pH-metry scores and decrease medication usage[63]. The success rate for implantation is 93% while the procedural success rate was reported at 98.7%[64]. Pooled results for Gatekeeper are shown in Table 1.

After completion of a 6 mo pilot study[65] with favorable results, a European multicenter study was initiated[66]. Patients underwent manometry, endoscopy, 24-hr pH-metry and symptom scoring before and after the procedure. The average number of prostheses implanted was 4.3 (2-6). Final results showed significant improvement in symptom scores (HRQL score from 24 to 5), quality of life, pH parameters (% time pH < 4, 9.1% to 6.1%) and LESP (8.8 to 13.8 mmHg) at 6 mo[64]. The prosthesis retention was 70% at 6 mo. Other studies with smaller numbers of patients have failed to show significant improvement in LESP, however, symptom scores and pH results show consistent improvement[63,66].

An international, prospective multi-center (US/European) randomized, sham-controlled Gatekeeper trial has recently commenced[67]. One hundred and forty four patients with symptomatic GERD dependent on PPI with evidence of reflux on 24-h pH study and a symptom score of > 20 off medications are being included in the study. Patients with Barretts esophagus, hiatal hernia > 3 cm, esophagitis (garde II or higher), stricture formation, varices or prior antireflux surgery will be excluded. Randomization will be attempted with a sealed envelope at a rate of 2:1 implant *vs* sham. Four implants will be placed circumferentially in the distal LES/cardia with re-treatment offered to individuals if GERD symptoms persist. Sham patients will be offered Gatekeeper therapy at 6 mo. Patients will take anti-secretory medications on a PRN basis. Outcome parameters are HRQL, esophagitis, medication use, 24 hr pH monitoring and implant persistence. To date, 52 subjects have been enrolled and randomized, 21 of whom have been implanted. Enrollment completion is anticipated in April, 2005[68].

Mechanism of action

The mechanism of action is probably similar to Enteryx in addition to bulking. The prosthesis narrows the lumen as seen at 6 mo follow-up endoscopy and is self contained.

Complications

The complications reported at 6 mo follow-up are shown in Table 2[64]. In the largest multicenter study, 2 out of 40 patients (5%) developed severe complications which included esophageal perforation caused by overtube placement and severe postprandial nausea (1 wk post-procedure) leading to endoscopic removal of the prosthesis at 3 wk[64].

Advantages

The Gatekeeper prosthesis is removable by endoscopic means. A needle knife can be used to incise over the edge of the implant which is then suctioned from its submucosal pocket[62]. Endoscopic ultrasound may be used for exact localization of the prosthesis. Of note, one of the two patients who had the prostheses removed was 7 mo post-procedure. All 3 prostheses were removed in the other patient, 3 wk post-procedure. No complication was encountered with either patient.

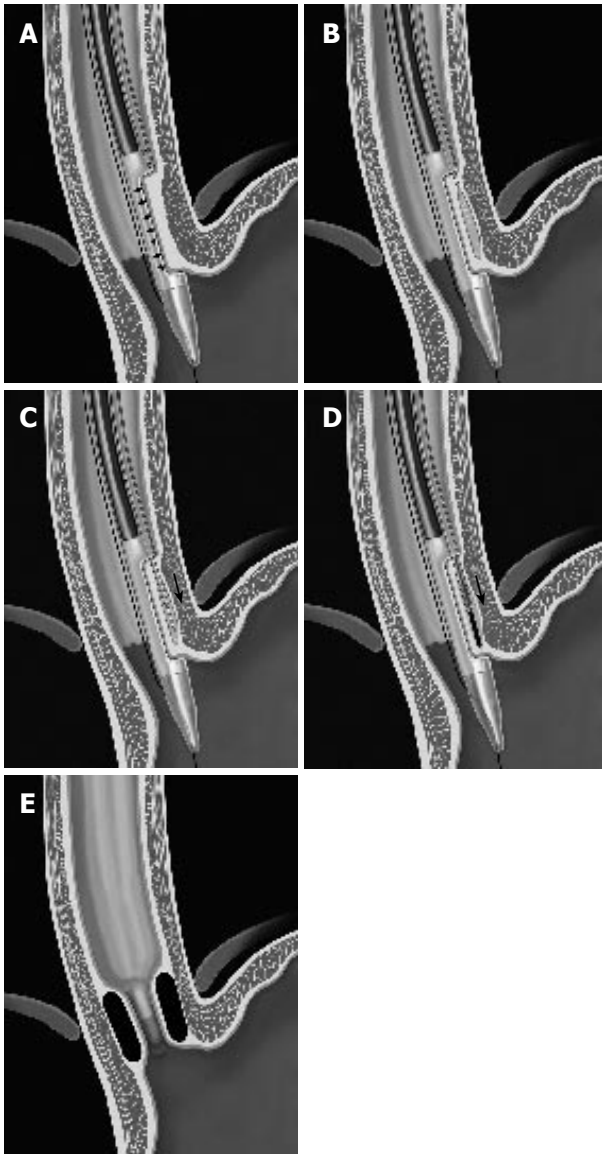
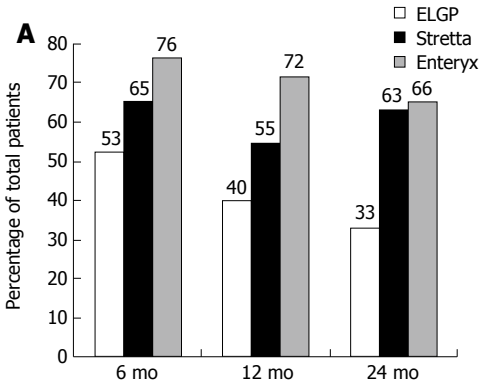


Figure 7 Gatekeeper procedure. Sequence of the procedure: **A.** Stabilization **B.** Create space **C.** Access space **D.** Delivery **E.** Gatekeeper.

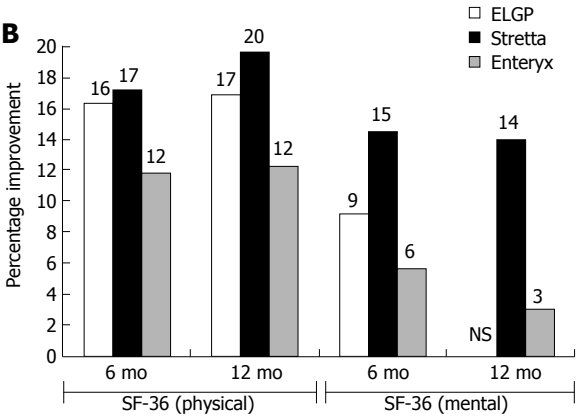
PLEXIGLASS

A trial of gelatinous plexiglass (polymethylmethacrylate, PMMA) microsphere implants has been published by Feretis *et al*[69]. A mean volume of 32 mL was implanted submucosally, 1-2 cm proximal to the squamocolumnar junction, in 10 patients with a 21-gauge needle. Transient dysphagia was noted in one patient due to excessive implant volume. At a mean follow-up of 7.2 mo, there was significant improvement of GERD-related symptoms and 24-h pH-studies (decreased from 24.5 to 7.2) but pH normalization was not seen. Ninety percent of patients were completely off PPI's at 6 mo follow-up. The procedure was found to be safe at short term follow-up.

Minor and self-limited complications occurred in 4 of 10 patients (40%). Transient dysphagia and the gas-bloat syndrome (10%) were thought to be due to excessive treatment with an implantation volume of 39 mL. The plexiglass injection was not associated with local or systemic complications and is not antigenic. Polymethylmethacrylate (PMMA) is highly viscous and

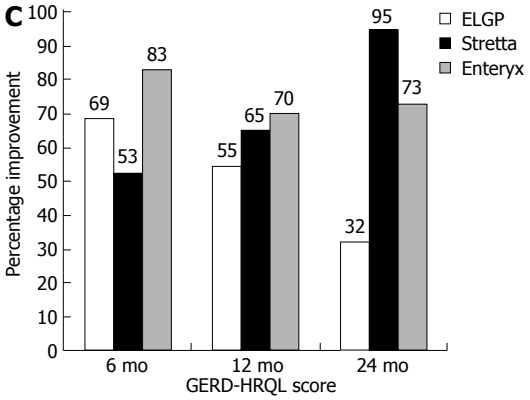


Graph showing percentage of total patients completely off PPI medication. Comparison of ELGP, Stretta and Enteryx at various follow-up intervals is shown. All changes are significant when compared to baseline. Pooled data was obtained from following references: 18, 20, 21, 23, 26, 31, 39, 41, 44, 45, 47, 49, 58, 74, 77, 82, 84.

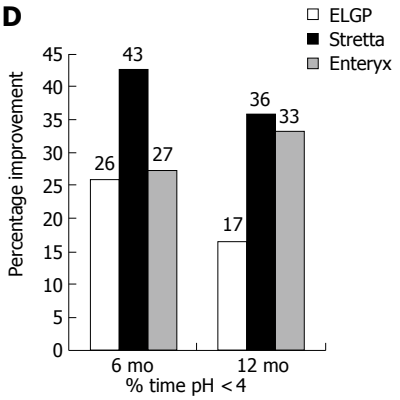


Graph showing the degree of improvement in quality of life assessment scores (SF-36) in patients with ELGP, Stretta and Enteryx at various follow-up intervals. NS, not significant. All other changes are significant when compared to baseline. Pooled data was obtained from the following references: 17, 21, 45, 46, 47, 58, 59, 74.

Figure 8 A: Percentage of total patients off PPI's at 6, 12 and 24 mo post-procedure; **B:** Improvement in quality of life scores (SF-36), both physical and mental, at various follow-up intervals; **C:** Degree of symptomatic improvement at various follow-up intervals; **D:** Improvement in % time pH <4 at 6- and 12- mo follow-up.



Graph showing the degree of improvement of symptom scores in patients with ELGP, Stretta and Enteryx at various follow-up intervals. All changes are significant when compared to baseline. Pooled data was obtained from the following references: 17, 18, 20, 21, 23, 26, 41, 45, 46, 47, 57, 58, 74, 77, 78, 80, 81, 82, 83.



Graph showing the degree of improvement of pH scores in patients with ELGP, Stretta and Enteryx at various follow-up intervals. Only studies showing significant improvement in pH scores are considered. Pooled data was obtained from the following references: 17, 21, 39, 45, 47, 58, 74, 79, 81, 83.

therefore a sigmoidoscope with a larger biopsy channel that accommodates a large caliber catheter was used for implantation. Longer follow-up studies are needed[69]. A multicenter study is currently being planned.

In addition, to these bulk-forming implants, injection therapy with sclerosing agents which induce focal necrosis and fibrosis similar have been tried. Sodium Morrhuate was used in 15 refractory GERD patients but after a year of follow up, the authors concluded that the therapy was ineffective[70].

COMBINATION THERAPIES

The use of endoscopic therapies after a failed fundoplication has been seen with ELGP, Stretta and Enteryx and their preliminary results have been discussed above. A combination of two different endoscopic therapies may be a solution to the lack of durability and objective benefit seen with these procedures. Anderson reported results of a pilot study done on 5 patients who were symptomatic and had radiological or pH probe evidence of reflux after prior ELGP procedure. These patients underwent Enteryx implantation and 3 of the 5 patients were off medication (60%) at a mean follow up of 7.6 mo. Transient dysphagia was seen after implantation in two patients but no major adverse effects were observed. Larger studies with longer follow up and objective documentation of improvement will help elucidate the efficacy of the combination approach[71].

Summary

The endpoints studied in most of the trials are GERD symptoms scores (HRQL), medication usage, manometric findings, grade of esophagitis and 24 hr pH-study results. In general, the procedures are safe with 3 deaths in 9000-10 000 cases. At present, the overall complication rate reported for ELGP, Stretta, Enteryx and Gatekeeper is 11%, 6%, 6.7% and 15% respectively[64]. All studies, to date, allow use of PPI's and most gauge success by the number of patients decreasing PPI dosage and symptomatic improvement. Meaningful conclusions cannot be made in this instance. There is evidence of symptomatic relief with decreased medication usage but with failure of an increase in LES length and pressure, healing of esophagitis and improvement in pH scores. An overview of the procedure for different endotherapies for GERD is shown in Table 2. A comparison of results in patients with Endocinch, Stretta and Enteryx at various follow-up intervals is also depicted in graphic format (Figures 8A-D).

In conclusion the scientific community needs to wait

for industry-independent trials showing endoscopic and 24 hour pH monitoring follow-up data that establishes long term efficacy and prolonged symptomatic benefit. Cost-effectiveness and post-marketing documentation of complications will better define the "true" role of these procedures in the management of GERD patients.

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EDITORIAL

Reassessment of functional dyspepsia: A topic review

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Abstract

Dyspepsia itself is not a diagnosis but stands for a constellation of symptoms referable to the upper gastrointestinal tract. It consists of a variable combination of symptoms including abdominal pain or discomfort, postprandial fullness, abdominal bloating, early satiety, nausea, vomiting, heartburn and acid regurgitation. Patients with heartburn and acid regurgitation invariably have gastroesophageal reflux disease and should be distinguished from those with dyspepsia. There is a substantial group of patients who do not have a definite structural or biochemical cause for their symptoms and are considered to be suffering from functional dyspepsia (FD). Gastrointestinal motor abnormalities, altered visceral sensation, dysfunctional central nervous system-enteral nervous system (CNS-ENS) integration and psychosocial factors have all being identified as important pathophysiological correlates. It can be considered as a biopsychosocial disorder with dysregulation of the brain-gut axis being central in origin of disease. FD can be categorized into different subgroups based on the predominant single symptom identified by the patient. This subgroup classification can assist us in deciding the appropriate symptomatic treatment for the patient.

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Key words: Dyspepsia; Epidemiology; *H pylori*; Subgroups; Cholecystokinin; Visceral hypersensitivity; Psychosocial; Central receptors; Therapy

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Functional dyspepsia (FD) is a heterogeneous disorder of yet unknown etiology. It is commonly encountered both in the primary practice as well as the gastroenterological outpatient clinic. Though never life threatening, FD has important social and economic implications. It is an important cause of job absenteeism and a major burden on health care services. The review article by Sanjiv and Goh[1] in this topic highlights reported that 20% of patients would have consulted either a general practitioner or a gastroenterologist, 50% would be on medications most of the time, 30% would have days off work or schooling and there is a definite reduction in the quality of life. They also maintain that proper epidemiological studies on dyspepsia is plagued with problems without agreed definitions on "dyspepsia", but the recent definition based on the Rome II working committee report has helped. It is also important to distinguish between the patients who present with dyspepsia that is uninvestigated (UD) and the true functional dyspeptics. Prevalence rate of UD varies across the world depending on the definition of dyspepsia used. When Rome II criteria were used, studies showed that approximately 25% of people in the community complain of UD. For FD the rates are still quite variable depending on the geographical background, varying from 11.5% to 29.2%.

Majority of patients with a diagnosis of FD continue to be symptomatic over long period of time despite periods of remission. Approximately one-third of the patients lose their symptoms spontaneously. Only some dyspeptics present for medical care, in the United States and United Kingdom 1 in 4 will consult but the figure is higher in Australia. Severity of pain and anxiety over the possibility of serious diseases are factors associated with consulting behaviour. In their review the authors also considered various risk factors that may be associated with UD or FD. It appears that being a female and having underlying psychological disturbances will predispose one to having FD. On the other hand, smoking, caffeine intake, poor socioeconomic status and NSAID (non-steroidal anti-inflammatory drugs) ingestion placed one at risk from developing UD.

Does the ubiquitous bacterium, *Helicobacter Pylori* (*H pylori*) have any role to play in FD? Most authors will agree that the evidence is still unclear and most *H pylori* eradication trials in FD have been badly designed and gave conflicting results. However in the reviews by both O'Morain and Malfertheiner[2,9], evidence were provided of well conducted randomized control trials and meta-analysis, showing a small but significant effect in eradicating *H pylori* in dyspeptic patients. O'Morain further suggested that *H pylori* associated dyspepsia is related to acid secretion. *H pylori* infection resulted in increased fasting and post-prandial serum gastrin levels and decreased gastric mucosal levels

of somatostatin. Of more significance is the fact that these abnormalities were corrected following eradication therapy. Furthermore, an intravenous infusion of gastrin-releasing peptide causes a six-fold increase in acid secretion in *H pylori* peptic ulcer patients, a four-fold acid increase in *H pylori* FD patients and a two-and-a-half fold increase in asymptomatic *H pylori* infected individuals, compared to asymptomatic controls without *H pylori* infection.

Most authors would agree that, *H pylori* when discovered needs to be eradicated to prevent gastric ulcer and reduce the risk of gastric cancer. It will be interesting to see in the long term, if the eradication of *H pylori* does any harm. Recently it has been suggested that eradicating the bacteria in susceptible individuals, may predisposed them to develop reflux oesophagitis , which can lead to Barrett's oesophagus and oesophageal carcinoma. Whether it is cost-effective to conduct a "test and treat" or "search and treat" strategy and on which population is still debatable. A lot of it will depend on the resources available to the individual community.

Interestingly, it has also been reported that *H pylori* FD patients are less likely to show evidence of dysmotility or delayed gastric emptying. However it has consistently been shown that approximately 50% of FD patients have some kinds of gastric motility disorder, the most common of which is delayed gastric emptying. Doubts still remain as to the relationship between gastric dysmotility and dyspeptic symptoms. Symptoms may be caused as a direct result of the abnormal motility or arises secondarily from the effect of the abnormal gastrointestinal propulsion, or a combination of both.

The subdivision of dyspepsia into subsets according to symptoms clusters remain controversial but are widely practiced[3]. Two main subgroups were widely recognized: "ulcer-like dyspepsia" and "dysmotility-like dyspepsia". Ulcer-like dyspeptic patients usually complain of upper abdominal pain while dysmotility-like dyspeptic have symptoms very suggestive of impaired gastroduodenal motility. However it has now being realized that the subgroup classification according to symptom clusters suggested thus far is of no clinical utility. More recent suggestions for a new classification is based on the most bothersome or prominent symptom presented by the patient. Newer trials seem to support the existence of such grouping in that severe postprandial fullness and vomiting were independently associated with delayed gastric emptying, while impaired gastric accommodation is linked to early satiety. Furthermore response to proton-pump inhibitors was better in the subgroup of patients with epigastric pain rather than discomfort. However there is also published data that does not agree with the above mentioned observation. This new sub-grouping will need to be assessed further before it can be widely used. Patients who have heartburn as their predominant symptoms are generally accepted to have gastro-oesophageal reflux disease and not dyspepsia. They may have erosive or non-erosive oesophagitis and even "functional heartburn". Similarly patients with lower gastrointestinal symptoms probably fall in the domain of irritable bowel syndrome (IBS) rather than FD.

A "hypersensitive" gut is now considered as the main pathophysiological factor underlying the functional bowel disorders. Patients suffering from the irritable bowel syndrome (IBS) have a lower threshold for pain especially during endoscopy and digital examination. Similarly FD patients demonstrate an increased sensitivity to gastric distension at lower distending pressures when compared to normal healthy controls. Other investigators have also shown that patients with FD respond abnormally to an infusion of lipid into the duodenum. The observed abnormality was due to the effect of CCK released locally causing gastric sensorimotor dysfunction. CCK's influence is exerted either locally on CCK mucosal receptors or at a distance on CCK receptors located on sensory afferent fibers or CCK receptors located in central circuits of the CNS. However it is also conceivable that the effect of the CCK is not from the increased level of circulating CCK but rather due to an altered response of the CCK receptors. In this respect we have demonstrated that FD patients respond abnormally to an infusion of CCK octapeptide[5]. CCK infusion reproduced the dyspeptic symptoms which can be blocked by atropine and loxiglumide. End organs (sensory receptors) present in the mucosa, muscle layer or serosa may respond abnormally (hypersensitive) to an external stimuli. The enhanced sensory perception may also result from an alteration of ascending sensory afferent neurons (vagal or spinal afferents) and perhaps from central amplification of visceral signals. Interestingly the presence of CCK receptors has also been demonstrated at all the above sites.

Keohane *et al*[4] suggested in their review that apart from visceral hypersensitivity, visceral hyperalgesia and viscero-somatic referral also play important roles in FD. In viscero-somatic referral the offending stimulus will elicit pain at a site distant from the site of application. Visceral hyperalgesia is the phenomenon whereby the application of an ordinary innocent stimuli result in pain. Hyperalgesia consists of both peripheral and central components, initiation and continuance of which contribute to the observed altered sensations that is so prominent in FD. It is possible that an acute visceral insult may recruit or sensitize sensory receptors or fibers, which even after the insult has subsided, will result in long term consequences of sensory-motor disturbances so characteristic of FD. It is proposed that altered mucosal mechano- or chemoreceptor may contribute to an exaggerated response to the CNS, when exposed to the presence of normal food content. Conceptually CCK and serotonin will be released from endocrine cells in the presence of normal luminal contents, which then act on their respective receptors to generate an abnormal response which when presented to a normal functioning CNS may be interpreted as inappropriately painful.

The CNS-ENS (brain-gut axis) interaction is vital in the pathophysiology of FD. As suggested by O'Mahony *et al*[5] and Chua[6] in their review, the dyspeptic symptoms may result from altered interactions at any level of the brain-gut axis. The CNS plays a central role in conducting and processing visceral signals. Alterations in brain processing of pain, perception and affective responses may be important in the pathogenesis of dyspeptic symptoms. The finely regulated motor, sensory and secretory activities of the GI tract are coordinated by the interactive actions between the CNS, autonomic system (sympathetic and parasympa-

thetic) and the ENS. Both the central serotonergic (5HT) and adrenergic (AD) systems play important roles in this interaction.

Central serotonergic pathways are important in the control of nociception. Depending on the receptor subtype activated, the response could be pro or antinociceptive. The antinociceptive effects of serotonin are dependent on noradrenaline being release by the serotonin. Similarly central adrenergic receptors play an essential role in the regulation of gastrointestinal motility. Activation of central alpha-2 (NAD) receptors mediates the inhibition of GI function. NAD neuronal input to the paraventricular and supraoptic nuclei in the hypothalamus arises from cell groups in the brainstem, all of which receive directly or indirectly visceral sensory input from the vagus and glossopharyngeal nerves. The paraventricular nucleus has output nerves to the brainstem and spinal cord which are important in regulating feeding and GI function.

Peripherally serotonin is well recognized for its effects on GI motility and secretion. Whether central serotonergic systems play the same role remains unclear. 5HT neurons in the pons and medulla contribute to the innervations of the paraventricular nucleus which influence autonomic and neuroendocrine function. The paraventricular nucleus also projects to the dorsal motor nucleus where vagal efferent neurons that control the stomach are located. Interestingly it has been shown that hypersensitive central 5HT receptors are associated with delayed gastric emptying and an abnormal response to CCK-8 infusion in FD. Furthermore the dyspeptic symptoms in FD can be reproduced by the CCK-8 infusion. The hypersensitive CCK receptors as mentioned earlier, under physiological condition of CCK release, may be stimulated and activate a vago-vagal reflex pathway that results in GI sensory-motor dysfunction and feeding abnormality.

FD has been shown to demonstrate hypersensitive central 5HT receptors while their central alpha-2 (NAD) receptors appear to be down regulated. Serotonergic and noradrenergic pathways may have opposite effects on feeding behavior and GI sensory-motor function. NAD stimulation of the paraventricular nucleus elicits feeding behavior that can be antagonized by local pretreatment with serotonin. The anorexic effect of CCK is dependent on central serotonergic pathways. It is possible that both the serotonergic and noradrenergic pathways may mediate the CCK effect in FD in an antagonistic way, serotonergic pathways being excitatory while the noradrenergic pathways are inhibitory. The observed sensory-motor abnormalities may be the result of a final pathway that originated in the sensitized peripheral receptors and afferent vagal pathway, modulated centrally, and the efferent pathways from the dorsal motor nucleus results in perturbation of peripheral GI function.

A biopsychosocial model to explain FD has been proposed, whereby biological, psychological and social factors interact to account for patient's symptoms, behavioral response and disease outcome. The brain gut axis plays a central role in mediating this interaction. FD can then be seen as a result of dysregulation of intestinal motor, sensory and CNS activity, resulting from interruptions at some level of the brain gut axis.

Stress, a commonly seen factor in precipitating symptoms in FD, may aggravate symptoms by its effects on the CNS. Acute or short term stress may result in delayed gastric emptying or other sensory-motor disturbances which are present in FD. Psychosocial factors as suggested by Dinan *et al*[7] in this topical review may be implicated in the predisposition, exacerbation and perpetuation of functional bowel disorder (FBD) including FD. Different types of stressors may play different roles in the pathophysiology of FBD. Early life stress and acute life-threatening situations are strong risk factors for developing FBD in the genetically predisposed individual, later on in life. Other stressors occurring throughout life transiently may cause intermittent changes in the stress response so as to cause symptoms exacerbation. Conceivably, psychological stress may affect peripheral sensory-motor function through an effect on central processing circuits. Similarly physical stress such as infection and trauma may contribute to and aggravate symptoms, and in the vulnerable to perpetuate symptoms.

Corticotrophin-releasing factor (CRF) has long been believed to have a physiological role in the mediation of CNS response to stress. CRF is also involved in reactions that coordinate and modulate the autonomic, behavioral, and visceral responses of the CNS to stress. Dinan *et al* suggested that CRF may act via central connections known as the emotional motor system (EMS). Ascending monoaminergic projections and circulating glucocorticoids has a feedback control on output fibers from the EMS that modulate a peripheral response which involves the neuroendocrine, autonomic and endogenous pain control pathways.

A number of psychological factors have been linked to FD. These includes psychological stress, personality traits, social support, life-events and life-stresses including abuse and bereavement. Barry *et al* have elegantly debated through each factors and their association to FD in their comprehensive review. The suggestion that psychological morbidity and social stressors merely motivating health care seeking measures has been challenged.

Similar to patients with FD, individuals with IBS have a higher rate of psychiatric comorbidities. The two entities though regarded as separate disorders have many similarities and overlapping symptoms. Both seem to have a genetic predisposition, prior infection or inflammation may contribute to its pathophysiology, while stress, life events and history of previous abuse play important roles in the progress and manifestation of these two chronic diseases. It is relatively easy for IBS patients with upper abdominal pain to be misdiagnosed as FD. This problem seems to be greater in Asia as Asians tend to present more commonly abdominal pain, according to Gwee *et al*[8]. In his review, one important reason for differentiating IBS from dyspeptic symptoms is to avoid unnecessary surgery. In patients with concomitant FD and IBS (FD-IBS), the risk of cholecystectomy is much higher compared to either FD or IBS alone. One interesting fact highlighted by Gwee *et al* is the observation that overlapping FD is more commonly seen in IBS with constipation. Moreover delayed gastric emptying has been reported in IBS patients with concomitant FD but not in those with IBS alone. Further-

more it has been shown that dysmotility-type FD is more likely to be associated with constipation type IBS. Though impaired accommodation to a meal is as prevalent in FD alone compared to FD-IBS, visceral hypersensitivity is significantly higher in FD-IBS patients. Post infectious IBS has generally being accepted as an entity while recent reports also suggested that FD could develop post-infection with delayed gastric emptying and impaired accommodation. The infectious agent could be viral in origin while a recent study reported dyspeptic symptoms post salmonella gastroenteritis.

FD does coexist with IBS and a substantial proportion of FD patients will evolve into IBS with time. Whether it is important to differentiate between FD and IBS remain to be seen. Clinically it is common practice to use combination treatment for both, suggesting that perhaps considering them as a single disorder may be more practical.

The response to drug treatment remains variable and uncertain. Monkemuller *et al*[9] in their review suggested an approach that deals with patients' predominant symptom. If the predominant symptom is epigastric pain or presence of gastroesophageal reflux, then an acid suppressive therapy should be considered with the proton-pump blockers. If *H pylori* is present, it should be eradicated. If the main symptom suggest dysmotility type FD then prokinetic agents can be considered. Cisapride and domperidone has been widely used and quite effective, until cisapride was removed due to cardiac side-effects. Other prokinetics such as erythromycin, tegaserod and alosetron has produced inconsistent results. Newer agents including itopride hydrochloride (ganaton) and mosapride may provide better results. A recent multicentre randomized controlled trial was published in the NEJM by Holtman et al showing the effectiveness of Itopride in relieving symptoms in FD. Loxiglumide, a CCK receptor antagonist has been shown in a small study to be effective in dysmotility-like dyspepsia, but this needs to be repeated in a bigger randomized controlled study. Antidepressants like the serotonin re-uptake inhibitors (SSRI) and amitryptalline will be useful in treating FD with psychological overlays. The success of this treatment strategy may also be attributable to the effects of these drugs on central pathways. The SSRIs act on central as well as peripheral 5-HT receptors while amitryptalline effects may be *via* central alpha-2 receptors. Hypnotherapy is effective in IBS but its role in FD remains to be seen. It is not widely available and very time consuming. Most studies looking at drug therapy in FD are plagued by the high placebo response. This could be accounted for by the significantly greater attention and time given to the patient by the researchers, with the attending investigations carried out per study protocol which could alleviate to a certain degree some of the anxiety, uncertainty or fear which the patients may have.

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TOPIC HIGHLIGHT

Andrew Seng Boon Chua, MD, *Series Editor*

Epidemiology of functional dyspepsia: A global perspective

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Abstract

Dyspepsia refers to group of upper gastrointestinal symptoms that occur commonly in adults. Dyspepsia is known to result from organic causes, but the majority of patients suffer from non-ulcer or functional dyspepsia. Epidemiological data from population-based studies of various geographical locations have been reviewed, as they provide more realistic information. Population-based studies on true functional dyspepsia (FD) are few, due to the logistic difficulties of excluding structural disease in large numbers of people. Globally, the prevalence of uninvestigated dyspepsia (UD) varies between 7%-45%, depending on definition used and geographical location, whilst the prevalence of FD has been noted to vary between 11%-29.2%. Risk factors for FD have been shown to include females and underlying psychological disturbances, whilst environmental/ lifestyle habits such as poor socio-economic status, smoking, increased caffeine intake and ingestion of non-steroidal anti-inflammatory drugs appear to be more relevant to UD. It is clear that dyspepsia and FD in particular are common conditions globally, affecting most populations, regardless of location.

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Key words: Dyspepsia; Uninvestigated dyspepsia; Functional dyspepsia; Prevalence; Epidemiology; Geographical variation

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INTRODUCTION

Dyspepsia refers to a collection of upper gastrointestinal symptoms that is believed to be common world-wide. Despite numerous consensus meetings[1-4], a standardized international definition that is applicable to all populations remains controversial. This is partly due to the overlap with gastro-esophageal reflux disease and the fact that cultural differences remain in reporting of symptoms[5]. The generally accepted definition by most clinicians includes the presence of upper abdominal pain or discomfort with or without other upper gastrointestinal symptoms, such as nausea, belching, vomiting, etc. However, confusion over what patients interpret as abdominal pain versus discomfort continue to "muddy the waters" in this issue[6].

Regardless of its definition, the causes of dyspepsia are known to include peptic ulcer disease, gastro-esophageal reflux, and functional dyspepsia. Functional dyspepsia, otherwise known as non-ulcer dyspepsia, is clearly the commonest cause of dyspeptic symptoms in the West and increasingly in other parts of the world[7]. The latest definition of this includes the presence of "chronic or recurrent pain or discomfort centred in the upper abdomen in the absence of any known structural cause and without any features of irritable bowel syndrome"[4]. The precise pathophysiology of this condition remains unclear, but it is thought to result from a combination of visceral hypersensitivity, gastric motor dysfunction and psychological factors[8].

Functional dyspepsia is not life-threatening and it has not been shown to be associated with any increase in mortality. However, the impact of this condition on patients and health care services has been shown to be considerable. In a recent community survey of several European and North American populations, 20% of people with dyspeptic symptoms had consulted either primary care physicians or hospital specialists, more than 50% of dyspepsia sufferers were on medication most of the time and approximately 30% of dyspeptics reported taking days off work or schooling due to their symptoms[9]. Similar findings have been reported by other leading investigators in this field[10], including the fact that people with functional dyspepsia have a significantly reduced quality of life when compared to the general population[11].

This review aims to describe the epidemiology of func-

tional dyspepsia from a global perspective. Previous reviews on this subject have been based mostly on published reports from Western populations[11,12], mainly as a result of a lack of data from non-Western countries. However, there has been a growth of population-based studies from Asia in the last few years and data from these publications will be emphasised in this review.

POPULATION-BASED STUDIES

In an attempt to demonstrate the variations between populations, we have decided to compare and contrast the prevalence and epidemiology of uninvestigated and functional dyspepsia from various geographical locations. Only studies that have been published in the English medical literature have been selected for this review. Population-based surveys, as opposed to referral-based (for endoscopy) or self-reporting studies, are more representative of the general population as issues such as selection bias (of consulters *vs* non-consulters) are minimized, although not completely excluded. In situations where different population studies from similar or close geographical locations have been presented separately, but have had similar results or conclusions, data from the larger cohort only will be examined in this review.

PREVALENCE OF DYSPEPSIA

The prevalence of dyspepsia varies considerably between different populations (Figure 1). Although these may represent genuine epidemiological differences, it is also apparent that the varying definitions used in different population studies may have contributed to this discrepancy. In studies using "upper abdominal pain" as the definition, the prevalence of uninvestigated dyspepsia (UD) has varied between 7%-34.2%[13,14,22-25,31,32,36]. With this definition, the lowest UD prevalence of 7%-8% is seen in Singapore, South East Asia[31], slightly higher rates are seen amongst the Scandinavians (14.5%[22] and 18.4%)[23,24], prevalence rates of 23-25.8% are seen in the US[13,14] with populations in India (30.4%)[32] and New Zealand (34.2%)[36] having the highest rates.

When a broader definition of "upper gastrointestinal symptoms" is used to define dyspepsia, a 23%-45% prevalence of UD[16-21,26,37] is observed. Using this definition, a lower prevalence is seen in Spain (23.9%)[26], a 32% UD prevalence rate in the US is noted[16], whilst significantly higher rates of 38%-41% are noted in the UK[18-21] and 45% in Nigeria[37].

In population studies that have used the Rome I criteria to define uninvestigated dyspepsia, a prevalence between 18%-38% has been observed[15,29-30,34-35]. The lowest prevalence of 18.4% was recorded in Hong Kong[30], whilst higher rates of 26% and 27.8% were noted in US[15] and Taiwan[29] respectively, and the highest prevalences of up to 38.2% were observed in populations in Australia[34,35].

Finally, with use of the recent Rome II criteria, where symptoms of reflux and IBS are excluded, surveys have reported prevalences around 24%[28,35]. Population studies in Australia and China reported prevalence rates of 24.4%[35] and 23.5%[28] of uninvestigated dyspepsia.

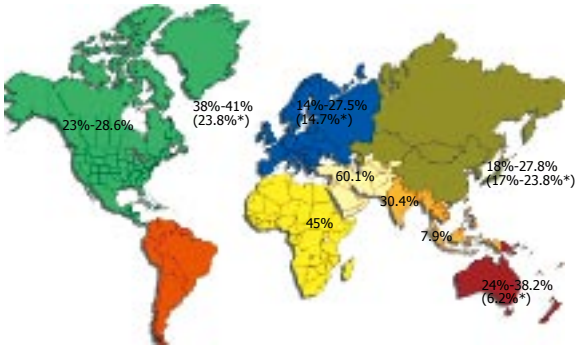


Figure 1 Global prevalence of uninvestigated dyspepsia and functional dyspepsia*.

True functional dyspepsia (FD), where organic disease has been excluded, has been difficult to determine in population studies, for obvious logistical difficulties. Nevertheless, several studies around the globe have actually undertaken this task to provide a reflection of the prevalence of functional or non-ulcer dyspepsia. In a survey of employees within a single institution in the US, Shaib and El-Serag managed to endoscope half of the survey participants and obtained a FD prevalence rate of 29.2% (with reflux symptoms) and 15% (without reflux symptoms)[16]. In the UK, two separate population surveys attempted to estimate the prevalence of FD. One fifth of 9936 subjects surveyed by Jones et al had been investigated with either a Barium meal or endoscopy, and an extrapolated FD prevalence of 23.8% was obtained[18]. Later, in a community survey of 2066 adults, 20% had undergone endoscopy in the study and FD was estimated at 11.5% of this population[19]. A 14.7% of FD was recorded in a Norwegian survey, where the majority of 2027 adults agreed to undergo endoscopy[25]. In Japan, Hirakawa et al were able to document a 17% prevalence of FD in adults undergoing a population gastric cancer screening programme[27]. Finally in Taiwan, the prevalence of FD appeared to vary, depending on criteria used. Of 2018 adults endoscoped, FD was documented at 23.8% with Rome I criteria and confusingly at 11.8% using the Rome II criteria[29].

EPIDEMIOLOGICAL FACTORS

Population-based studies determining the prevalence of dyspepsia have attempted to identify epidemiological risk factors for UD, and when relavant FD as well. Below is a brief summary of various parameters that have been studied in association with the prevalence of dyspepsia.

Age

All surveys that have been conducted have examined adults 18 years or older. While most surveys have shown that dyspepsia does not appear to be related to any particular age group, several studies have noted some trends. Peak prevalences of UD have been noted between the ages 45-54 in a Canadian survey[17], whilst FD appeared to peak in Chinese subjects 41-50 years[28] and in Japanese adults 50-59 years[27]. In the latter study, dyspepsia sub-types appeared to be associated with different age groups: reflux-like more common in middle-aged adults, dysmotility-like more

frequent in those < 59 years and ulcer-like predominant symptoms more frequently in adults < 39 years. In other populations, the prevalence of UD appeared to decrease with increasing age in British[18], Taiwanese[29] and Danish[23] surveys. In the latter survey, there was a significantly lower prevalence of UD in adults > 70 years (10%) compared to those < 60 years (18.4%)[23]. In contrast, a survey in urban Mumbai, India found that UD was more prevalent in adults > 40 years[32]. Despite these trends, age extremities has not been identified as a predictor of dyspepsia (UD or FD).

Gender

Most population studies have been able to obtain relatively equal ratios of male: female ratios and the majority of them have shown no differences in dyspepsia prevalence between genders, mostly where UD is concerned. Several studies, in different populations, however, have noted a consistent female preponderance with dyspepsia[16, 23, 26, 29,34]. Female gender was found to be the only independent risk factor for FD amongst 2018 Taiwanese health check attendees[29]. In a population-based study in Australia, female adults significantly outnumbered males in most functional GI disorders, including FD[34]. As only a few population studies have examined true FD prevalence, it is likely that the gender effect in surveys of UD have been masked due to the combination of adults with FD and organic dyspepsia.

Ethnicity

The role of ethnicity in dyspepsia has not been examined by most population studies. Most surveys have been done on populations of single/similar ethnic groups, mostly of Caucasian or Oriental background (Table 1). However, in one of the few studies involving subjects of several ethnic backgrounds from a single institution in the US, African-American race was found to be one of several epidemiological risk factors for UD[16]. In a survey of a multi-racial population in Singapore, South East Asia, the ethnic-adjusted prevalence of UD was demonstrated as follows: Chinese 8.1%, Malays 7.3% and Indians 7.5%[31]. Although the majority ethnic group in Singapore is Chinese, the authors were able to obtain prevalence based on equal representations of the three different ethnic groups. At present, little can be concluded regarding the role of ethnicity and it is clear that more data is required from future studies.

Smoking

Although a common practice world-wide, regular smoking as a risk factor has not been consistent in its association with dyspepsia. In the few population-based studies that have examined FD, smoking has not been shown to be a risk factor[25,28,29,34]. In surveys of patients with UD however, regular smoking has been identified as a risk factor in populations in US[16], Canada[17], UK[21] and India[32]. This observation may be explained by the proportion of organic disease amongst subjects with UD, as smoking has been identified as clear risk factors for diseases like peptic ulcer disease[38].

Alcohol

Regular alcohol intake, as a risk factor, has been studied and it has not been shown to be associated with dyspepsia in the vast majority of surveys. However, in the Asia-Pacific region, only population studies in India[32] and New Zealand[36] have showed definite associations between alcohol and UD.

Non-steroidal anti-inflammatory drugs

The effect of non-steroidal anti-inflammatory drugs (NSAIDs) on dyspeptic symptoms have been examined specifically in only two population-based studies. In a survey of American adults from a single institution, regular usage of NSAIDs and Aspirin, bought over the counter, were strongly associated with UD than in controls without dyspepsia[16]. In a British study of 4982 adults, NSAID usage was identified as an independent risk factor for UD and thought to be responsible solely for 4% of dyspepsia in the community[21]. Interestingly, data from the African sub-continent may correlate this fact in a study of Nigerian highlanders. "Indulgence in self-medication" amongst the subjects surveyed was found to be a significant risk factor for UD[37]. Although this was not described clearly, and probably included various types of traditional medication, it is probable that analgesics containing NSAIDs may account for a sizeable amount of this "self-medication".

Helicobacter pylori infection

To date, only one population-based study in the UK has investigated the association of *H pylori* infection with UD. Among 8047 subjects who were tested for *H pylori,* those who were infected had more dyspeptic symptoms (44%) than those who were *H pylori* negative (36%)[21]. Subsequent analysis revealed *H pylori* status to be predictive of UD and the authors concluded that *H pylori* infection had a 5% population attributable risk for dyspepsia assuming a causal association. The association of *H pylori* and FD is less clear, but this has only been examined in some detail in non-population-based studies[39].

Dietary factors

The role of diet in dyspepsia has not been studied by many, probably due to the diversity of dietary habits within individual populations. In the few studies that have attempted to examine dietary factors and their association with dyspepsia, the definitions of food types and categories do not appear to be clear. In the Chinese study examining the prevalence of FD[28], "bad dietary habits" was shown to be a significant risk factor. However, the authors fail to clarify their definition of this term. In an urban survey in India, Shah et al managed to demonstrate that no differences in dyspeptic symptoms occurred between vegetarians (29.1%) and meat-eaters (31.2%), whilst spicy, fried or food prepared outside the home contributed insignificantly to worsening of symptoms[32]. In Nigerian adults living in the highlands, the type of staple food consumed was strongly associated with UD, but no specific definitions of food types are given[37]. The effect of caffeine intake has also been examined in some population studies,

Table 1 Epidemiology of uninvestigated dyspepsia (UD) and functional dyspepsia (FD)

Location	*n*	Population	Ethnicity	Age (yr)	Definit on of dyspepsia	Prevalence UD	Prevalence FD	Gender difference	Risk factors
N. America									
US [13]	1021	One county	Caucasian	30-64	Upper abdominal pain	25.8%	NA	None	NA
US[14]	919	One county	Caucasian	30-49	Upper abdominal pain	23%	NA	None	Sexual abuse Physical abuse
US[15]	8250	National	Caucasian	49.1 (mean)	Rome I	26%	2.7%	None	< 45 yr age Lower income Being employed
US[16]	465	One institution	Black 46% Caucasian 36%	44.6 (mean)	Upper GI symptoms	31.9%	29.2%	Yes (F > M)	Black ethnicity NSAIDs Smoking
Canada[17]	1036	12 Cities	Not stated	18-80	Upper GI symptoms	28.6%	NA	None	Low socio-economic status Life events Smoking Cola
Europe									
UK[18]	9936	National	Caucasian	20-69	Upper GI symptoms	41%	23.8% (estimate)	None	NA
UK[19]	2066	2 Communities	Caucasian	20-80	Upper GI symptoms	38%	11.5%	None	NA
UK[21]	9262	2 counties	Not stated	40-49	Epigastric pain ± upper GI symptoms	38%	NA	None	Low socio-economic status *H pylori* infection Smoking
Europe									
Sweden[22]	1422	One municipality	Caucasian	20-87	Upper abdominal pain	14.5%	NA	None	NA
Denmark[23,24]	4581	One county	Caucasian	30-60	Epigastric pain	16.2% (M) 20.8% (F)	NA	Yes (F > M)	Psychological disorder Social problems
Norway[25]	2027	One municipality	Caucasian	20-69	Upper abdominal pain	27.5%	14.7%	None	Family history Tranquiliser usage
Spain[26]	284	One city	Not stated	18-80	Upper GI symptoms	23.9%	NA	Yes (F > M)	None
Asia									
Japan[27]	911	1 city	Japanese	18-70	Upper GI symptoms	NA	17%	None	NA
China[28]	1016	Health check attendees	Chinese	15-75	Rome II	NA	23.5%	None	Poor diet Low socio-economic status Societal pressure Past abuse Anxiety/depression
Taiwan[29]	2018	Health check attendees	Chinese	18-80	Rome I Rome II	27.8%	23.8% 11.8%	Yes	Female gender
Hong Kong[30]	1649	National	Chinese	37.9 (mean)	Rome I	18.4%	NA	None	Anxiety/depression
Singapore[31]	706	1 Community	Chinese 8.1% Malays 7.3% Indians 7.5%	21-95	Upper abdominal pain ± upper GI symptoms	7.9%	NA	None	NA
India[32]	2549	One city	Indian	18-80	Upper abdominal pain	30.4%	NA	None	Alcohol Smoking
Jordan[33]	2254	One county	Arabic	> 16 yr	Not stated	60.1%	NA	None	NA
Australasia									
Australia[34]	2910	One city	Not stated	43.8 (mean)	Rome I	38.2%	6.2%	None	Female gender Anxiety/ depression
Australia[35]	2300	One city	Not stated	> 18	Rome I Rome II	32.5% 24.4%	NA	None	NA
New Zealand[36]	952	community	Not stated	> 18	Recurrent upper abdominal pain	34.2%	NA	None	Alcohol Aspirin use
Africa									
Nigeria[37]	1151	community	African		Upper GI symptoms	45%	NA	None	Family size Occupational scatter Type of food Melaena Self medication

particularly from Western studies. Surveys in the US and Europe have reported that excessive coffee or tea intake has not been shown to be related to the presence of dyspepsia/UD[16,17,21,23,25]. However, in one of the few studies to examine its' role, a Canadian survey showed that heavy intake of cola was associated with markedly increased prevalence of dyspepsia[17]. An explanation for this observation may be due to the fact that greater quantities of caffeine in cola can be consumed more readily, or it may be a non-caffeine related compound which is responsible for dyspeptic symptoms. Clearly, more studies on the role of diet in dyspepsia are needed, but standardization in dietary terminology and habits are required before meaningful conclusions can be elicited from such data.

Socio-economic associations

Most population-based studies have examined basic socio-demographic associations in dyspepsia and the majority have not revealed any significant findings, eg between social classes and prevalence of dyspepsia. However studies examining details of socio-economic status were able to elicit associations with dyspepsia. Drossman in the US noted a strong relationship between lower household income and larger household membership with increased functional GI diseases, including FD[15]. Similarly, a Canadian survey revealed that chronic GI symptoms (UD) were more prevalent in adults with lower household income, those who were unemployed and with lower educational levels[17]. In a British survey, factors including rented accommodation, no central heating, low educational level and sharing a bed with siblings (surrogate for crowded household) were found to be predictive of UD in adults[21]. Amongst an urban population of dyspeptics in China, "dissatisfaction with financial income" was associated with FD, but this was not as significant as other psychological factors (see below)[28]. Finally, in the Nigerian study, a larger sized family together with occupational scatter was strongly associated with UD[37].

Psychological associations

In most population surveys that have studied psychological disturbances as a risk factor, definite risk associations, particularly for FD, have been elicited. Talley *et al* had previously reported in an American adult population that sexual, emotional and verbal abuse either in childhood or adulthood were significantly associated with dyspepsia[14]. This, in turn resulted in more health-care seeking behaviour amongst this group of adults. In a Danish survey, Kay and Jorgensen noted that UD was strongly associated with adults who had "experience of problems" and "psychological vulnerability"[23]. In one of the few population-based studies that managed to examine FD in some detail (by excluding structural abnormalities in most of the adults), the authors found that FD patients, as opposed to those with UD alone had a significant association with tranquiliser usage[25], probably a surrogate marker for anxiety or a neurotic behaviour. This observation is similarly observed in an Australian survey where adults with FD scored highly on anxiety and depression scales[34], and in a Chinese study which revealed "pressure from society" and "destructive living habit" as risk factors for FD[28]. Yet another survey in Hong Kong also revealed that subjects with UD had more anxiety, compared to adults with IBS, which appeared to influence health-care seeking habits[30].

Summary

Table 1 summarises the features of the epidemiology of dyspepsia from a geographical perspective. The published data to date supports the notion that dyspepsia is common in most populations of this world. The varying prevalence of UD in different populations, some even in similar geographical locations, appear to be related to the different definitions of dyspepsia used by investigators of individual surveys. The true prevalence and epidemiology of FD amongst the general population has not been evaluated as much, due to the difficulties in excluding organic disease in large numbers of people. Nevertheless, several studies[16,25,27,29] have been able to examine this in some detail. With the data that is available, the true prevalence of FD globally is estimated between 11.5%-29.2% (including symptoms of reflux) (Figure 1). Epidemiologically, it appears that risk factors for FD are different to that of organic dyspepsia and even general UD. Where this has been studied in some detail, female gender and underlying psychological disturbances have been shown to be important factors in FD[16,25,28,29,34]. In contrast, environmental/lifestyle habits such as poor socio-economic status, smoking, increased caffeine intake and NSAID ingestion appear to be more relevant to UD[17,21,28,32] . We believe this is a result of the presence of organic disease in these populations. Although a peak preponderance of dyspepsia around the middle ages (40-50 years) have been reported in some surveys[26,27], the extremities of age do not appear to be predictive of dyspepsia generally. Apart from one study from the US[16], the role of ethnicity has not been shown to be relevant. However, most of the published surveys have usually been on single/majority ethnic populations and hence this factor has yet to be refuted for certain.

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TOPIC HIGHLIGHT

Andrew Seng Boon Chua, MD, *Series Editor*

Subtypes of functional dyspepsia

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Abstract

Functional dyspepsia is a common clinical condition characterised by chronic or recurrent upper abdominal pain or discomfort commonly associated with a variety of associated gastrointestinal symptoms and a normal endoscopy. To standardise research-based approaches, an initial categorisation of into sub groups was agreed to, based on clusters of symptoms. However the early expectation that these subgroups would be associated with distinct pathophysiologies amenable to specific therapy has not been realised. A classification based on the most troublesome symptom has been suggested but the utility of this is also unclear. More recent data suggest that some of the pathophysiologic dysfunctions may be associated with specific symptoms and so provide a better tool for grouping patients. But this approach remains incomplete as current insights into the pathogenesis are still too limited for this to be satisfactory. In conclusion, no classification provides for an adequate treatment-based approach to the syndrome of functional dyspepsia. As a consequence treatment remains largely empiric.

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Key words: Functional dyspepsia; Classification; Subtypes; Symptoms; Pathophysiology; Management

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INTRODUCTION

Dyspepsia is broadly defined by predominantly midline pain or discomfort located in the upper abdomen[1]. Although the term often implies a relationship with food ingestion and majority of patients have symptoms worsened by food[2] this is no longer essential. Dyspeptic symptoms occur commonly in the general community[3] and constitute one of the most frequent reasons for referral to gastroenterologists[4]. Surveys suggest as many as 15%-20% of the population in Western countries experience dyspepsia over the course of one year[5]. During investigation of dyspepsia, three major structural causes are readily identifiable: peptic ulcer disease (10%), gastroesophageal reflux (20%)(with or without oesophagitis), and malignancy (2%)[6]. It is apparent that most (50%-70%) patients with chronic dyspepsia do not have a significant focal or structural lesion found at endoscopy. When symptoms are chronic or recurrent, but without an identifiable structural cause using standard diagnostic tests (usually endoscopy), the condition is usually labelled functional (or non-ulcer) dyspepsia[7]. Hence functional dyspepsia is a diagnosis of exclusion that implies that symptoms have been investigated without the demonstration of an organic or anatomical cause.

INITIAL APPROACHES TO CLASSIFICATION INTO SUBTYPES

To standardise research studies amongst this heterogenous population of patients, a definition of functional dyspepsia was developed by consensus amongst a group of international experts. In addition to describing the nature of reported symptoms a temporal component of persistent or recurrent for 4 wk in a 3 mo period was included[1]. This was subsequently modified to include more than 12 wk in one year[8]. As many patients with a final diagnosis of functional dyspepsia presented with symptoms suggestive of specific diseases initial efforts to subcategorise the syndrome, proposed subdividing dyspepsia into symptom-based subgroups namely reflux-like, ulcer-like, dysmotility like and non-specified[1]. The underlying expectation was that such subgroups would share common pathophysiology and might also respond to specific therapy. However, as is typical of a chronic condition, the manifestations of dyspepsia can be complex. Patients with upper abdominal discomfort frequently complain of a multiple of related symptoms. (Table 1)[9]. Indeed population-based studies indicate that patients presenting with uninvestigated dyspepsia, have at least three dyspepsia symptoms; more than 80% have at least six and approximately half have eight or more[10].

Table 1 Definitions of dyspeptic symptoms

Symptom	Description
Pain centred in the upper abdomen	A subjective unpleasant, patients may feel that tissue damage is occurring. Other symptoms may be extremely bothersome A subjective unpleasant without being interpreted as pain.
Discomfort centred in the upper abdomen	A subjective unpleasant sensation that is not interpreted as pain, which may include any of the symptoms below.
Early satiety	A feeling the stomach is overfilled soon after starting to eat, out of proportion to the size of the meal.
Fullness	An unpleasant sensation of the persistence of food in the stomach (may or may not occur post prandially).
Bloating in the upper abdomen	A tightness in the upper abdomen, this should be distinguished from true abdominal distension.
Nausea	A feeling of the need to vomit.
Retching	Heaving as if to vomit but no gastric contents are forced up.

Furthermore, symptoms often vary over time. The initial classifications were expanded to include cluster analysis of these associated symptoms.

With further intensive investigation, it has become apparent that dividing patients with uninvestigated dyspepsia empirically according to clusters of symptoms has not assisted in identifying specific gastrointestinal mechanisms for different symptom types[11]. This may well reflect the extensive overlap of symptoms within the empirically derived groupings[12] the frequent association with irritable bowel syndrome[13] and the temporal variation in symptoms[9, 12, 13]. It also became apparent that the symptom sub classification was a poor predictor of underlying structural abnormality.[14]

SUB-TYPES IN FUNCTIONAL DYSPEPSIA POST ROME II (TABLE 2)

Concerns about the lack of progress in understanding the syndrome and the overlap between various symptom clusters resulted in a refinement of these in a subsequent consensus agreement[8]. In particular the inclusion of patients with predominant symptoms of GORD was considered unwarranted as this entity should be distinguished in its own right. Thus the ROME II assessment of Functional Gastrointestinal Disorders recognised three subtypes of functional dyspepsia[8]. (1) Ulcer-like: Characterised by predominantly epigastric pain or discomfort which may worsen with hunger and improve with food or antacids. As with ulcer disease, symptoms may remit and relapse spontaneously. (2) Dysmotility-like: Contained clinical features suggestive of disturbed upper gastrointestinal motility. Thus upper abdominal discomfort aggravated by food commonly associated with nausea, vomiting, bloating and upper satiety together. Anorexia also occurred in part because of the consequences of food ingestion was also a feature and often associated with significant weight loss. (3) Unspecified: Patients with symptoms which did not fit either of the categories above.

Table 2 Categorisation of Adult Functional Gastroenterological disorders as per Rome II[1]

A: Oesophageal disorders
- Globus
- Rumination syndrome
- Functional chest pain of presumed oesophageal origin
- Functional heartburn
- Functional dysphagia
- Unexplained functional oesophageal disorder

B: Gastroduodenal disorders
- Functional dyspepsia
- Ulcer-like dyspepsia
- Dysmotility-like dyspepsia
- Unspecified (non-specific dyspepsia)
- Aerophagia
- Functional vomiting

C: Bowel Disorders
- Irritable bowel syndrome
- Functional abdominal bloating
- Functional constipation
- Functional diarrhoea
- Unspecified functional disorder

D: Functional abdominal pain
- Functional abdominal pain syndrome
- Unspecified abdominal pain syndrome

E: Biliary Disorders
- Gall bladder dysfunction
- Sphincter of Oddi dysfunction

F: Anorectal disorders
- Functional faecal incontinence
- Functional ano-rectal pain
- Levator ani syndrome
- Proctalgia Fugax
- Pelvic floor dyssynergia

As noted previously, patients with functional dyspepsia often do not fit neatly into symptom cluster classifications, in part because of the wide overlap of symptoms[13]. The attempts to subgroup patients using the Rome II modification has also not been particularly helpful in either clinical practice or research studies[11]. In addition the substantial overlap between dyspeptic groups, those with GORD and other functional gastrointestinal disorders has limited the utility of this approach (Figure 1)[15] although it has been suggested that functional dyspepsia can usually be separated from symptoms related to the lower gut (ie Irritable Bowel Syndrome) by the presence of bowel disturbance despite the fact that many patients had both dyspepsia and IBS[16]. In the light of the extensive symptom overlap and poor discriminatory value in individual patients, the value of the cluster-based symptom classification has been questioned and possibly replaced by a subcategorisation based on the most troublesome symptom. The value of this approach remains unproven[17]. Notwithstanding these restrictions, some recent data suggest that symptom clusters based around specific pathophysiologic disturbances may provide insights into the mechanisms of the functional dyspepsia[2]. These appear to have predictive value and could possibly direct approaches to the development of novel strategies.

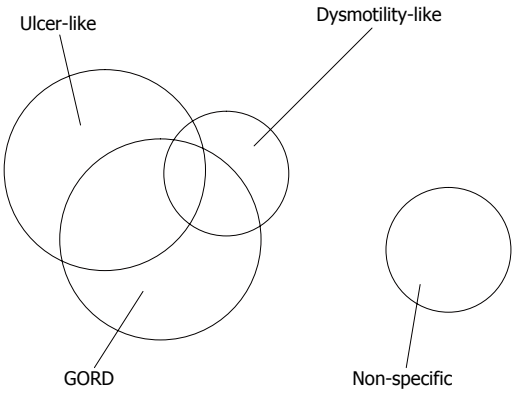


Figure 1 Schema of overlap of dyspepsia subtypes (according to Rome II) and gastro-oesophageal reflux disease[15].

GORD and functional dyspepsia

It has become obvious that it is important to discriminate between symptoms related to GORD and functional dyspepsia. A sensation of retrosternal or epigastric burning that characteristically radiates towards the throat, is precipitated by meals or the supine posture and is relieved at least temporarily by antacids, is strongly suggestive of GORD[15]. Frequent heartburn increases the likelihood of GORD as the cause of symptoms[6] although pyrhosis may be much less obvious in the older patient[18]. It is likely that the majority of dyspeptic patients with predominantly reflux symptoms have GORD, although only 50% of these patients have oesophagitis at endoscopy. As noted above, the revised criteria in ROME II did not include reflux-like dyspepsia as a sub-grouping of functional dyspepsia, and patients with heartburn as their major symptom are now generally excluded from dyspepsia studies. However it needs to be recognized that many patients with GORD have multiple symptoms[10] and a substantial number of patients with GORD also have functional dyspepsia which may be unmasked by successful treatment of GORD with PPI. A number of patients with functional dyspepsia may have heartburn as an extra symptom and the prevalence of GORD in patients with epigastric pain alone is unknown. It is also unclear whether this could represent undiagnosed or non-erosive reflux disease.

Pathophysiologically based sub-groups

The pathophysiology of functional dyspepsia is unclear and a large number of mechanisms proposed as causative[19]. These include delayed gastric emptying[20,21], visceral hypersensitivity[22], impaired gastric accommodation to meals[23], abnormal motility[24], infection with *Helicobacter pylori* (*H pylori*)[25] and CNS dysfunction. In addition, childhood abuse[26] and prior gastrointestinal infection[27] have been described as precursors to later dyspepsia. However, an association between the majority of these mechanisms and specific symptoms is lacking.

CLASSIFICATION ACCORDING TO SPECIFIC PATHOPHYSIOLOGIES ASSOCIATED WITH FUNCTIONAL DYSPEPSIA

Gastric motor and sensory dysfunction

Although it has long been recognized that gastric emptying is delayed in approximately 1/3 of patients with functional dyspepsia[20], this does not reliably predict individual patient symptoms, with the exception that there is some association with fullness and bloating[28, 29]. Gastric antral hypomotility measured by manometry is well described[24], but may well reflect the limitations of pressure recordings with non-lumen occlusive contractions. More promising are recent descriptions of the proximal gastric function, which may permit better discrimination of some symptoms related to meal ingestion[2]. Thus intolerance to gastric distension suggesting impaired visceral sensitivity has been associated with epigastric pain, and belching[22]. Similarly, impaired proximal gastric accommodation has been associated with pain, early satiety and weight loss particularly in young women[23]. Of particular interest to clinicians, are reports that these abnormalities can be evaluated with a high calorie drink test[30]. This technique potentially allows an office-based approach to address at least some symptoms in these patients, without resorting to invasive gastric barostat techniques, or expensive technology with limited availability[31-33].

Helicobacter pylori Infection

Depending on the population under study, between 30%-65% of patients diagnosed with functional dyspepsia have *H pylori*-induced gastritis[25,34]. However, a consistent causal link between *H pylori* infection and specific symptom profiles has not reliably been established[35]. *H pylori* positive individuals are as likely to have symptoms of bloating and early satiety as symptoms suggestive of peptic ulcer[17]. Suggestions that *H pylori* infection has a significant impact on gastric motility have also not been confirmed[36]. It is feasible that some of these patients could have undiagnosed peptic ulcer disease. At present, the aetiology of symptoms in *H pylori* patients without peptic ulceration is unclear[37].

Intestinal hypersensitivity

Dyspeptic symptoms are frequently worsened by food especially those rich in fat. A heightened sensitivity to both lipids[38] and acid[39] have been reported to be possible mechanisms underlying symptoms. High fat meals slow gastric emptying and hence may accentuate bloating and fullness. A similar effect may be seen with high fibre meals. These studies involved small numbers of patients and their general applicability at this stage remains uncertain.

CLASSIFICATION ACCORDING TO POSSIBLE PATHOGENESIS

The pathogenesis of functional dyspepsia is unknown and probably multi-factorial. Only a minority of patients report previous gastrointestinal infection[27] or an acute onset compatible with a post-infective aetiology. Psychological factors especially stress[40] and previous abuse[26] have been suggested to be important but direct evidence for a causative role is yet to be established. A recent description of an association between dyspeptic symptoms and a functional polymorphism of a G-protein subunit[41] has demonstrated that patients who are homozygous for the GNB3 825C subunit have a higher incidence of abdominal symptoms. The full implications of this is currently under

investigation. In the light of these uncertainties, further data are required before a meaningful classification based on potential pathogenesis can be undertaken.

THERAPEUTIC IMPLICATIONS

Given the limited understanding of the pathophysiological basis for symptoms in functional dyspepsia, there are limited options for rational treatment. It appears however unlikely that a single therapy will effectively manage all or even most patients with the condition[42]. In addition to general measures such as reassurance and avoiding obvious precipitants, the most commonly available options are: (1) Eradication of *H pylori* infection; (2) Acid reducing therapy; (3) Gastrokinetic agents

Eradication of helicobacter pylori infection

In patients where abdominal pain is the most bothersome symptom especially if ulcer-like in character, current practice frequently involves eradication of *H pylori* particularly in regions where *H pylori* infection is endemic[42]. This has the advantage of treating patients where the diagnosis of peptic ulcer has been missed as well as reducing the risk of subsequent malignancy. These patients may well be cured, although it must be recognised that the overall direct therapeutic gain from this approach in dyspeptic patients in general is small[43].

Acid-reducing therapy

Although ulcer-like symptoms would be expected to benefit from acid suppression with simple antacids, H_2 receptor antagonists or more recently proton pump inhibitors, the therapeutic benefit is small[42,44]. Some of this may derive from successful management of hidden GORD and peptic ulcer disease.

Gastrokinetic drugs

Data indicating that patients who have predominant pain are associated with hypersensitivity to gastric distension suggests that targeting modulation of gastric accommodation (such as $5HT_1$ antagonists) may have a role in the future management[2] although such approaches are at present in limited use. In patients with dysmotility-like symptoms prokinetic therapy is a rational approach especially if supported by objective evidence of delayed gastric emptying or a positive response to a satiety drinking test. Cisapride, a substituted benzamide with activity as a $5HT_4$ agonist, was arguably the most effective prokinetic agent although the evidence for an effect on symptoms is controversial[42]. The drug has been withdrawn in many countries due to rare but life-threatening cardiac toxicity. Another 5-HT_4 agonist with prokinetic activity, mosapride[45], has not been widely released. Newer agents such as tegaserod[46] and itopride[47] are currently undergoing evaluation. In patients with *H pylori* gastritis there is the theoretical possibility that gastritis-induced hypersensitivity may be ameliorated by treating the infection[35].

In conclusion, the sub-classification of functional dyspepsia is currently in transition. The early expectation that particular subtypes based on dominant symptom clusters would be associated with specific pathophysiologic disturbances has not been realised, although these descriptive terms remain entrenched in clinical practice. More recent data support a division into pathophysiologically-based patient groupings. In particular there appears to be an association between slow gastric emptying and dysmotility-like symptoms whilst hypersensitivity to gastric distension is more common in patients with post prandial pain. Eradication of *H pylori* gastritis has limited direct therapeutic gain. Patients with predominantly reflux symptoms need exclusion of GORD. Until further data are available it seems rationale to base initial therapy on the suspected pathophysiological abnormalities rather than symptoms, although well evaluated therapeutic options are limited. Further research is required to clarify the relationship between symptoms and motor and sensory dysfunction to provide an evidence-based management strategy.

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TOPIC HIGHLIGHT

Andrew Seng Boon Chua, MD, *Series Editor*

Functional dyspepsia: The role of visceral hypersensitivity in its pathogenesis

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Abstract

Functional, or non-ulcer, dyspepsia (FD) is one of the most common reasons for referral to gastroenterologists. It is associated with significant morbidity and impaired quality of life. Many authorities believe that functional dyspepsia and irritable bowel syndrome represent part of the spectrum of the same disease process. The pathophysiology of FD remains unclear but several theories have been proposed including visceral hypersensitivity, gastric motor dysfunction, *Helicobacter pylori* infection and psychosocial factors. In this review, we look at the evidence, to date, for the role of visceral hypersensitivity in the aetiology of FD.

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Key words: Visceral hypersensitivity; Motor dysfunction; *Helicobacter pylori*; Psychosocial

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INTRODUCTION

Functional dyspepsia is a common clinical condition in the community and is one of the most common disorders encountered by gastroenterologists worldwide. It forms part of the functional gastrointestinal disorders (FGID's), a spectrum that includes irritable bowel syndrome (IBS), non-cardiac chest pain, and non-ulcer or functional dyspepsia and that overlaps to a significant extent with non-erosive reflux disease (NERD)[1]. Studies of FD prevalence have provided varied rates but one study estimated that as many as 25% of Americans may be affected[2]. However not all of those affected seek medical attention for their symptoms. While the individual symptoms may be included within the concept of FD are also varied and include post-prandial fullness, bloating, early satiety, nausea, and vomiting, the hallmark of functional dyspepsia is the presence of persistent or recurrent pain or discomfort centred in the upper abdomen for which there is no organic cause. According to the most widely employed definition of FD, Rome II, this symptom should have been present for at least 12 wk within the preceding 12 mo in order to be classified as FD[3]. The advent and widespread use of the Rome criteria has facilitated clinical studies, including studies of pathophysiology, by ensuring, as far as possible, the inclusion of a relatively homogenous population within any given study. In the evaluation of any study of the role of visceral hypersensitivity, or indeed, any other factor in the pathophysiology of FD, it is critical to be fully apprised, not only of the criteria that were employed in the definition of the study population, but also of their geographic origin and the location of the study. These and other features, such as age, socio-economic status and ethnicity, can lead to tremendous variations in the relative contributions of various factors to the pathophysiology of FD, in a given population. For decades, general practitioners and specialists alike dismissed these patients as malingerers or as having some form of somatization disorder. However, it is only in the last decade or so that attempts have been made to elucidate the true pathophysiology of this heterogeneous disease. A number of putative mechanisms have been elucidated, including visceral hypersensitivity, delayed gastric emptying, impaired gastric accommodation, acid sensitivity, *Helicobacter pylori* (*H pylori*) infection and disturbed central perception of peripheral visceral events.

With regards to the relative contributions of these factors, Stanghellini *et al* found delayed gastric emptying in 33% of FD patients, and this was associated with female gender, low body weight, the presence of severe postprandial fullness, and vomiting, and the absence of pain as a dominant symptom[4]. Impaired gastric accommodation is thought to be present in approximately 40% of patients and is associated with early satiety and weight loss[5-7]. As regards *H pylori*, its role in the generation of symptoms remains controversial but certainly there is evidence to show that persistent infection may alter gastric

motor and sensory physiology; however, the benefits of eradication remains somewhat controversial[8-10]. The prevalence of hypersensitivity to gastric distension has been reported to be of the order of 30%-40%. Attempts to link mechanisms with symptoms have met with mixed results; proposed symptom associations with various pathophysiologic mechanisms are outlined in Table 1 below[11].

Method

An extensive review of the role of visceral hypersensitivity in the pathogenesis of functional dyspepsia was undertaken. For this purpose a systematic search of Pubmed databases 1966-2003 for relevant literature was performed, using the following key word combinations: functional dyspepsia or non-ulcer dyspepsia and visceral hypersensitivity. Relevant literature was reviewed and cited accordingly.

Table 1 The mechanism and associated symptoms in functional dyspepsia

Mechanism	Associated symptoms
Delayed gastric emptying	Postprandial fullness, nausea, vomiting.
Hypersensitivity to gastric distension	Epigastric pain, belching, weight loss.
Impaired accommodation	Early satiety, weight loss.
Helicobacter infection	Epigastric pain
Duodenal lipid hypersensitivity	Nausea
Duodenal acid hypersensitivity	Nausea
Unsuppressed phasic contractility	Bloating, absence of nausea
Atypical nonerosive reflux disease	Epigastric pain

VISCERAL HYPERSENSITIVITY

In order to fully understand visceral hypersensitivity we must first return to neurophysiology and understand how stimulation of the gut wall results in conscious perception. The perception of somatic pain involves a three-neuron chain, as illustrated in Figure 1. For visceral pain, a similar mechanism is operative. The signal is initiated at sensory receptors in the mucosa, muscle layer or serosa and relayed *via* intrinsic primary afferent neurons (IPAN's), vagal or spinal sensory afferent neurons to second order neurons in the enteric nervous system, brain stem or spinal cord, respectively.

Theories abound regarding potential sites of abnormal sensory signalling in FD. While most studies have involved mechanical stimuli, such as distension, there is evidence of sensitivity to other stimuli. For example, Samsom and colleagues have previously shown that FD patients are more sensitive to the introduction of exogenous acid into the duodenum[12]. Firstly, the existence of hypersensitivity, at the level of gut mechanoreceptors, has been nicely illustrated by Tack and colleagues[13]. In this experiment, they took fifty patients with documented visceral hypersensitivity by barostat studies and treated half with fundus-relaxing drugs, sumatriptan and clonidine. When re-tested, those treated with gastric relaxing drugs now demonstrated a significant reduction in gastric sensitivity, thereby, illustrating the role of the mechanoreceptor in the generation of symptoms from a hollow viscus in the gastrointestinal tract, as had been previously shown by others, in healthy volunteers[13-15].

Much of the initial work on visceral hypersensitivity involved balloon distension of the stomach. The development of the barostat allowed investigators to measure gastric tone and sensory responses accurately and reproducibly for the first time[16]. It has long been known in clinical practice that patients with irritable bowel syndrome (IBS) have a low threshold for the development of pain when subjected to certain stimuli, such as digital rectal examination, sigmoidoscopy and colonoscopy. This was confirmed some time ago by balloon inflation studies in IBS[17]. More recent studies have suggested that both visceral hypersensitivity[18], and visceral hyperalgesia[19], the phenomenon whereby innocuous stimuli become painful, are highly specific for IBS. Another related phenomenon characteristic of IBS is that of viscero-somatic referral, whereby the pain elicited by a given stimulus is experienced at a site remote from that of its application.

Similar observations have been made in functional dyspepsia, thereby, advancing the visceral hypersensitivity hypothesis in this disorder also. Several studies have looked at balloon distension of the stomach using the gastric barostat and have all found increased sensitivity to distension at lower distending pressures relative to normal healthy controls[20-25]. Mearin and colleagues nicely illustrated this phenomenon. In their study, one can clearly see the distinct difference in abdominal discomfort scores between the FD patients and the control subjects as intragastric pressure is increased[22].

Mearin's group also, rather interestingly, showed that there were no differences in terms of somatosensory responses between controls and dyspeptics thus suggesting that this was truly a visceral phenomenon. Some would even suggest that visceral hypersensitivity to distension appears to be organ specific, with the response to rectal distension being specific for IBS and that to gastric distension being similarly specific for FD. However, a subsequent study by Bouin *et al* looked at somatic sensation by hand immersion in cold water, and found that those with functional gastrointestinal disorders perceived pain earlier and had a lower pain tolerance than normal controls[26]. These findings suggest, in contrast, that hypersensitivity is not just a visceral problem, but, perhaps, forms part of a more generalised sensory dysfunction. Whether differences in patient populations or testing methodology can explain these apparently discordant results remains unclear.

Mertz and colleagues compared sensory responses in a group of FD patients with both healthy controls and a group of patients with organic cause for their dyspepsia[24]. They examined both sensory thresholds to gastric balloon distension and the nature of the symptoms perceived during gastric distension. While there was no real difference in the prevalence of symptoms between the organic and functional groups (apart from nausea as a primary complaint being significantly more common in the FD group), only those with functional dyspepsia

had lowered perception thresholds to gastric distension, 87% versus 20%. In addition, they showed, rather nicely, the presence of viscerosomatic referral in the FD patient group alone. This could explain how patients with functional dyspepsia may perceive pain at sites distant to the stomach.

THE BRAIN-GUT AXIS

Other potential sites for sensory abnormalities in functional dyspepsia are the sensory afferent neurons, the spinal cord, and the brain itself. More and more research is now focusing on the so-called brain-gut axis. Normally, most visceral sensation to the central nervous system (CNS) does not reach conscious perception but patients with FD are thought to perceive visceral stimuli in an abnormal manner[27]. Vandenberghe and colleagues demonstrated, in their study, that visceral hypersensitivity may originate at sites other than the gut wall and involve a multimodal pathway[28]. They showed that both painful and non-painful gastric distension resulted in higher symptom scores among a group of FD patients with visceral hypersensitivity, yet gastric compliance was similar to that in a group of FD patients without hypersensitivity, suggesting that altered perception was more than a gut wall phenomenon.

Research in animal models has furthered our knowledge of the visceral sensory pathways (Figure1). Previously it had been thought that nocioception was mediated by spinal afferents and the sympathetic system[29], until animal studies established the role vagal afferents played in the modulation of nociception[30,31]. The advent of functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) scanning have greatly increased our knowledge of the final sensory pathways in the spinal column and the brain[32]. Two recent studies, in healthy volunteers, have demonstrated the activation of several areas of the brain in individuals subjected to gastric distension[32,33]. Some of the areas of activation included the thalamus, the insula, the left and right postcentral gyrus, and the anterior cingulate gyrus. The latter, interestingly, was previously shown to be an area of low activation, in IBS patients, in response to a painful stimulus[34]. Similarly, non-painful oesophageal balloon distension activates the primary somatosensory cortex, the insula bilaterally, and the operculum and painful stimuli activate the right anterior insular cortex and the cingulate gyrus[35,36]. These studies illustrate the complexity of central nervous system processing of visceral pain.

FOOD HYPERSENSITIVITY IN FUNCTIONAL DYSPEPSIA

Typically, and almost by definition, FD patients report the occurrence of most their symptoms in relation to food ingestion. Accordingly, studies have shown that about 60%-70% of patients with FD are hypersensitive to an infusion of lipid into the duodenum[37,38]. Another study found more dyspeptic symptoms in patients following the ingestion of high-fat versus low-fat soups[39]. It would also appear that hypersensitivity is nutrient specific as the intraduodenal infusion of an emulsion of long-chain triglycerides induced fullness, nausea, and bloating in patients with FD, whereas an infusion of glucose did not[40]. A role for fat was further illustrated in a recent study on healthy volunteers where the lipase inhibitor, orlistat, markedly reduced the perception of fullness and nausea induced by duodenal lipid infusion and gastric distension[41].

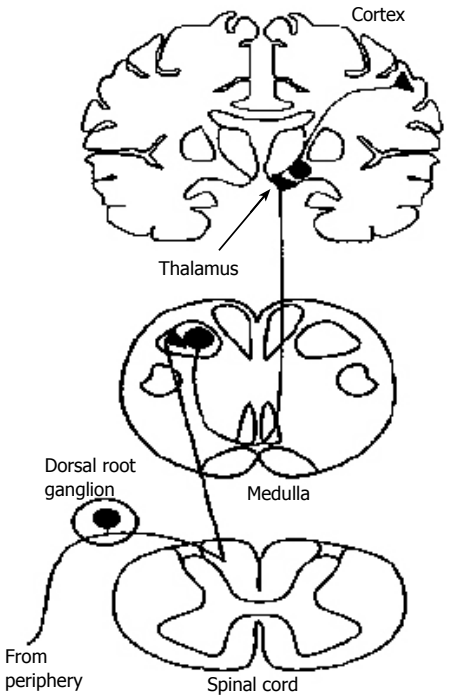


Figure 1 Sensory pathway showing one route of visceral sensation from the periphery, via a spinal sensory afferent neuron to the central nervous system.

Duodenal infusion of acid was also found by Samson and colleagues to induce nausea in FD; these patients also demonstrated impaired clearance of exogenous acid from the duodenum[12]. It is clear from these studies and others that small intestinal sensing of certain nutrients and fat, in particular, as well as the motor response in the foregut to their instillation, may play a role in the induction of symptoms in FD. In this way motor and sensory phenomena may interact and prove synergistic in symptom induction.

Helicobacter pylori

H pylori infection is purported in several studies as a possible mechanism in functional dyspepsia. However, recent large scale controlled trials and a meta-analysis failed to show any improvement in dyspeptic symptoms with *H pylori* eradication[42-44].It is unclear what role *H pylori* plays in the symptom profile of the FD patient, and whether any of the proposed pathophysiological mechanisms mentioned earlier are related to *H pylori* infection. These particular questions were addressed in a recent study by Sarnelli and colleagues[45] in which they compared the symptom profile of FD patients with and without *H pylori*, and looked at various pathophysiological mechanism including gastric emptying, sensation and accommodation.

They found no association between *H pylori* infection and the overall prevalence of symptoms or the gastric sensory-motor functions. This is in keeping with the previous observations that eradication of *H pylori* is not associated with with improvement in the dyspeptic symptoms.

In conclusion, clearly, a vast amount of knowledge regarding FD has been garnered from detailed studies of the last decade or so. The level of complexity involved in visceral sensation is only now being fully understood and therapies aimed at ameliorating the hypersensitive gut are crucial. These will involve visceral analgesics and agents that block the various neurotransmitters involved. The development of enhanced neuroimaging will allow us better understand the mechanisms of actions of some of these agents. Functional dyspepsia often leaves the clinician despondent due to the lack of clinical efficacy of many of the drugs used, but novel agents and targets of treatment offer hope for the treatment of this complex condition.

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TOPIC HIGHLIGHT

Andrew Seng Boon Chua, MD, *Series Editor*

Role of *Helicobacter pylori* in functional dyspepsia

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Abstract

The aetiology of dyspepsia is unknown in the majority of patients. *Helicobacter pylori* (*H pylori*) is the cause in a subset of patients. A non invasive test to assess the presence of *H pylori* is recommended in the management of patients under the age of 50 presenting to a family practitioner with dyspepsia. A urea breath test or a stool antigen test are the most reliable non invasive tests. Eradication of *H pylori* will reduce the risk to the patient with dyspepsia of developing a peptic ulcer, reduce the complication rate if prescribed non-steroid anti-inflammatory drugs and later reduce the risk of gastric cancer. The recommended treatment for non ulcer dyspepsia associated with a *H pylori* infection should be a 10-d course of treatment with a PPI and two antibiotics. Treatment efficacy should be assessed four weeks after completing treatment with a urea breath test or a stool antigen test.

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Key words: Functional dyspepsia; *Helicobacter pylori*; Triple therapy; Urea breath test

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INTRODUCTION

Dyspepsia or indigestion is an unsatisfactory term as it describes a spectrum of conditions. It has other limitations as there is no similar word in all languages. The patient does not volunteer symptoms and the diagnosis depends on a doctor's interpretation of the patient's complaint. Symptoms are vague and varied but for research and clinical purposes are grouped as reflux, ulcer like, motility or non specific[1].

Since the aetiology of dyspepsia is uncertain, it is not surprising that (*Helicobacter pylori*) *H pylori* has been implicated in dyspepsia. Large population studies have shown that the bacteria is found more frequently in dyspeptic patients compared to controls. Peptic ulcer relapse and remit and it is possible that at the time of endoscopy an ulcer is not present and then the patient is diagnosed with non ulcer dyspepsia as the bacteria will be present[2]. There is unequivocal evidence that infection with *H pylori* is the principal cause of peptic ulcer disease. The organism is present in 85%-95% of patients with peptic ulcer disease, and treating the infection is effective in healing these ulcers. Treatment to eradicate *H pylori* results in permanent cure of peptic ulcer disease, whereas 60%-80% of such patients relapse within a year if treated with anti-secretory drugs alone[3].

The evidence for an association between *H pylori* and non-ulcer dyspepsia is more uncertain. Many trials evaluating the efficacy of *H pylori* eradication treatment for non-ulcer dyspepsia have been poorly designed and have given conflicting results but there is a clear indication that *H pylori* eradication treatment is effective in at least a subset of patients with non-ulcer dyspepsia.

INVESTIGATING DYSPEPSIA

Patients presenting to a primary care physician with symptoms referable to the upper gastrointestinal tract are usually treated empirically with a proton pump inhibitor (PPI) or are assessed for the presence of *H pylori* by a non-invasive test. The recommended non invasive tests are a urea breath test or a stool antigen test. A serology test is considered less reliable and is not recommended. Consensus guidelines have recommended a test and treat approach to patients with *H pylori*[4,5]. This has been controversial as the response to treatment has been equivocal. In a double blind study the test and treat approach has been vindicated in non investigated dyspepsia in patients presenting to a family practitioner. In a study of 294 patients who were randomised to triple therapy of a PPI and two antibiotics, clarithromycin and metronidazole for one week or a PPI and placebo antibiotic the patients were evaluated monthly for one year[6]. The results of this randomised control led study demonstrate a significant benefit of a *H pylori* "test and treat" strategy at primary care level (Figure 1).

Endoscopy

Investigated dyspepsia describes patients who had an endoscopy and in whom no mucosal lesions are found, but are found to have an *H pylori* infection on biopsy. Systematic review of randomised controlled trials comparing *H pylori* eradication with placebo or another drug treatment has been published[7]. In this analysis the trial reports were reviewed according to predefined eligibility and quality criteria. Twelve trials were included in the systematic review, nine of which evaluated dyspepsia at 3-12 mo in 2541 patients. *H pylori* eradication treatment was significantly superior to placebo in treating non-ulcer dyspepsia (relative risk reduction 9% (95% confidence interval 4% to 14%)). One case of dyspepsia being cured for every 15 people treated. *H pylori* eradication cost £56 per dyspepsia-free month during first year after treatment. The conclusion of this comprehensive data review is that *H pylori* eradication is cost effective treatment for non-ulcer dyspepsia in infected patients.

Test and treat vs endoscopy

A number of economic models were developed suggesting that eradication models were developed suggesting that eradication of *H pylori* might be cost saving in dyspepsia. Silverstein *et al* reported that the 1 year medical charges for the initial management of an incident episode of dyspepsia were $2163 for prompt upper endoscopy versus $2123 for empirical therapy, a difference of only 2%; the decision was a toss-up across all age groups and clinical strata applying medical charges[8]. Other models support empirical *H pylori* therapy as being less costly. Fendrick *et al*[9] in a model restricted to persons with symptoms suggesting peptic ulcer disease, reported that the most expensive strategy was endoscopy and biopsy for *H pylori* at $1584, while the costs per patient treated were lowest for serologic testing for *H pylori* (and treating seropositive cases) at $894 and empirical anti-secretory therapy combined with antibiotics (for all cases) at $818. Only if an upper endoscopy cost $500 or less was an endoscopy strategy superior.

Ofman *et al*[10] in a model of *H pylori*-positive patients with dyspepsia, found that empirical treatment was less expensive ($820 *vs* $1276), largely due to less upper endoscopy (52%). They calculated that endoscopy related costs had to be reduced by 96% before initial endoscopy and *H pylori* treatment were similarly cost-effective. Other models have suggested benefits may be marginal[11] or will take at least 5 years to accrue[12]. Upper gastrointestinal radiology is still practiced in some primary care settings but was not a cost-effective alternative to a *H pylori* test and treat strategy. In another US model[13], the test and treat approach is cost effective compared to prompt endoscopy. The benefit is more apparent if there is a high prevalence of peptic ulcer and of *H pylori* infection in the population. The value of this approach decreases when the prevalence rate of the infection falls. The critical prevalence below which the test and treat is less effective although still an option is 15%[14].

The yield from endoscopy in patients being investigated for dyspepsia increases with advancing age but is low[15]. Missing early (and hence curable) gastric cancer is often of greatest concern to the clinician contemplating empirical therapy, especially in an older patient[16,17]. Fear of gastric cancer has to be taken into account when planning the management of dyspepsia. A practical approach to investigate patients with endoscopy presenting with dyspepsia should be made for alarm signs and not on the basis of age alone, reflecting the balance of benefit and harm from endoscopy. Empirical management without formal diagnosis is appropriate for most patients: reviewing the patient history, lifestyle, over-the-counter medicines, and providing a course of proton-pump inhibitors and/or *H pylori* test and treatment. Patients with ongoing symptoms require at least annual review to discuss symptoms and lifestyle, and as appropriate, encourage stepping down prescribed medication and returning to self-care. A new strategy included in the step down process is the use of therapies 'on-demand'.

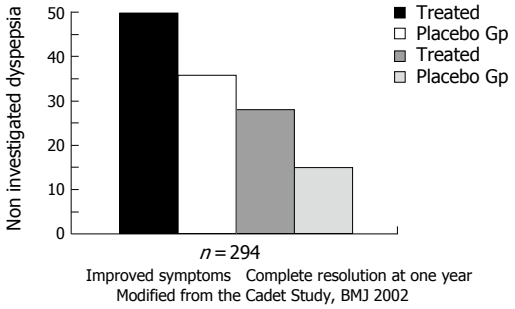


Figure 1 Significant improvements in complete resolution of symptoms in patients successfully treated of *H pylori* infection.

However, there is inconclusive evidence that patients without alarm signs will benefit subsequently from endosocpy, while investigation involves a small but real risk of harm[18].

Long term studies

The effectiveness of a test and treat strategy, is confirmed in long term studies. In two long term follow up studies of almost 7 years the benefits of eradication were still apparent[19,20]. In a short term prospective study from my unit of 90 patients with non ulcer dyspepsia, no clinical or endoscopic evidence of other peptic biliary, pancreatic or malignant disease; all had evidence of *H pylori* infection were randomised to bismuth alone, antibiotics alone or a combination of bismuth and antibiotics. Overall *H pylori* eradication was achieved in 49% of patients, although gastritis improved in those in whom the bacteria was eliminated whereas symptoms score were similar in patients where the infection persisted or was eradicated[21] when assessed at four weeks. However, a follow-up study at one year of these patients showed the patients in whom the bacteria persisted required additional treatment and in were more symptomatic compared to those who were eradicated of the bacteria.

The same patients when reviewed at a 5-yr follow up showed that patients who were successfully treated were more likely to be asymptomatic and none developed a

duodenal ulcer. Where as up to 20% developed an ulcer when the bacteria was still present (Figure 2)[22].

PATHOGENESIS

There is some evidence that *H pylori*-associated dyspepsia is caused by the effect of the bacterium on acid secretion[23]. *H pylori* infection causes increased fasting and post-prandial serum gastrin levels and decreased gastric mucosal concentrations of stomatostatin abnormalities which are corrected following eradication[24-27].

Gastrin-releasing peptide is a neuro-peptide that mimics the physiological responses of the stomach to a meal. After intravenous infusion of gastrin-releasing peptide, patients with peptic ulcers and *H pylori* infection have a six-fold increase in acid secretion and patients with non-ulcer dyspepsia and *H pylori* have a four-fold increase, whereas asymptomatic *H pylori* positive individuals have a two-and-a-half-fold increase in stimulated acid secretion, when compared to asymptomatic controls who do not have *H pylori* infection[28]. This would suggest that non-ulcer dyspepsia may represent part of a spectrum of *H pylori*-induced disease. The spectrum ranges from an asymptomatic carrier state, through an ulcer-free dyspeptic period to finally developing a peptic ulcer.

Further work from my unit suggest that mast cells are found in *H pylori* negative dyspeptic patients, compared to *H pylori* positive patients suggesting a different mechanism of symptoms in *H pylori* negative dyspepsia[29].

TREATMENT

It has been suggested that the eradication rates in patients with non ulcer dyspepsia is not as successful in patients with duodenal ulcer[30]. The possible explanation is that the bacteria colonisation is not as dense and that the gastritis is not as severe allowing poorer penetration of antibiotics. It emphasis the need for compliance. Smoking is associated with a lower success in treatment and may serve as surrogate marker for compliance. The treatment is complex and it is know that individuals who have received third level education achieve only 50% compliance to a five day course of antibiotics. Patient education is vital for successful treatment. They should be warned of possible side effects such as diarrhoea and antibiotic sensitivity. Diarrhoea is usually transient and self limiting and occasionally cases of clostridium difficile have been reported.

New options in the treatment armamentarium against *H pylori* provide hope of higher eradication rates. The use of a more prolonged 10-d course of therapy rather then the traditional 7-d course, and quadruple rather than triple therapy may lead to higher eradication rates. In a French study of 2571 patients, the use of 10-d therapy as opposed to 7-d therapy was associated with higher eradication rates- 83.9% compared with 70.6%[30]. A meta-analysis of 13 studies evaluated lengthening triple therapy beyond 7 d. Only randomised trails comparing 7-d triple therapy with either 10 or 14 d triple therapy were included. In the intention to treat analysis, pooled 10 and 14 d therapies lead to better results than 7 d schedules. However, in head-to-head comparisons, although a trend for higher cure rates was found with longer therapy, only 14-d therapies were found to be significantly better than 7-d therapy. Looking at intention-to-treat analysis, 10 d therapy compared with 7-d therapy lead to a 3% increased eradication rate-from 80 to 83% - whereas, 14-d therapy compared with 7-d therapy led to a 9% increased eradication rate from 72 to 81%[31].

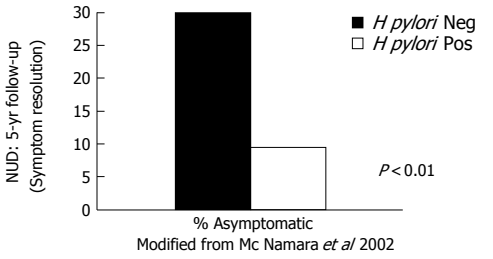


Figure 2 Patients who had *H pylori* eradicated were less symptomatic at 5 years compared to patients who had persistent *H pylori* infection.

CONCLUSION

A test and treat strategy is the preferred option for patients with dyspepsia presenting to primary carer physicians. The benefit of this are apparent in long term studies. The additional benefit of eradication of the bacteria will prevent subsequent development of an ulcer and the complication that might ensue if prescribed NSAIDs later and reduce the risk of gastric cancer. Treatment compliance is essential. The preferred treatment is a PPI based triple therapy, which should be given for a period of at least 10 d.

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TOPIC HIGHLIGHT

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Central serotonergic and noradrenergic receptors in functional dyspepsia

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Abstract

Functional dyspepsia is a symptom complex characterised by upper abdominal discomfort or pain, early satiety, motor abnormalities, abdominal bloating and nausea in the absence of organic disease. The central nervous system plays an important role in the conducting and processing of visceral signals. Alterations in brain processing of pain, perception and affective responses may be key factors in the pathogenesis of functional dyspepsia. Central serotonergic and noradrenergic receptor systems are involved in the processing of motor, sensory and secretory activities of the gastrointestinal tract. Visceral hypersensitivity is currently regarded as the mechanism responsible for both motor alterations and abdominal pain in functional dyspepsia. Some studies suggest that there are alterations in central serotonergic and noradrenergic systems which may partially explain some of the symptoms of functional dyspepsia. Alterations in the autonomic nervous system may be implicated in the motor abnormalities and increases in visceral sensitivity in these patients. Noradrenaline is the main neurotransmitter in the sympathetic nervous system and again alterations in the functioning of this system may lead to changes in motor function. Functional dyspepsia causes considerable burden on the patient and society. The pathophysiology of functional dyspepsia is not fully understood but alterations in central processing by the serotonergic and noradrenergic systems may provide plausible explanations for at least some of the symptoms and offer possible treatment targets for the future.

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Key words: Functional dyspepsia; Serotonin; Noradrenaline; Gastrointestinal disorders

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INTRODUCTION

Dyspepsia is defined as pain or discomfort centred in the upper abdomen[1]. Discomfort specifically includes early satiety, fullness, upper abdominal bloating and nausea[2]. It is a common symptom and comprises 30 to 40% of all abdominal complaints presenting to gastroenterologists[3]. Patients treated in primary care usually have uninvestigated dyspepsia, and their symptoms may have an underlying structural cause, such as peptic ulcer disease, reflux oesophagitis, or endoscopy negative gastro-oesophageal reflux disease. However, a large number of these patients will have functional dyspepsia (FD) where there is no structural or biochemical explanation for their symptoms following appropriate investigation[3].

FD may be categorised by the predominant symptom into ulcer-like, dysmotility-like, and unspecified dyspepsia. Ulcer-like dyspepsia has pain as the predominant symptom while dysmotility-like dyspepsia has predominantly discomfort. There was another category of dyspepsia, reflux-like dyspepsia, but this is no longer included as these patients have reflux disease and are treated as such. This classification scheme was first used as an aid in the design and performance of clinical trials. Its clinical use beyond taxonomy has yet to be proven[1,2].

FD is a heterogeneous disorder which does not have a well established pathophysiology. Gastrointestinal motor abnormalities, altered visceral sensation, and psychosocial factors have all been identified as major pathophysiological mechanisms. It has become more evident recently that FD is a biopsychosocial disorder[4] in which these three major pathophysiological mechanisms interact to generate the symptoms. This view has now replaced the earlier perspective that the condition was the result of a sole motor or sensory disorder of the stomach[5]. There is much controversy whether *Helicobacter pylori* infection induces FD or not. Some studies suggest that it is implicated in a small proportion of patients with FD[6] whilst others suggest that it is not involved in the pathophysiology at all[7].

The motor, sensory and secretory activities of the

intestine occur through the coordinated bidirectional communications between the central nervous system (brainstem and cerebral cortex), the autonomic nervous system (ANS) (sympathetic and parasympathetic neuronal pathways) and the enteric nervous system[8]. These systems form what is known as the brain-gut axis[9,10]. FD symptoms may result from deregulated interactions at any level of the brain-gut axis. Recent studies emphasize the role of the central nervous system (CNS) in conducting and processing visceral signals and suggest that alterations in brain processes involving perception and affective responses might be key factors in the pathogenesis of functional gastrointestinal symptoms[10]. Familial clustering of FD has been reported[11] which suggest a genetic component. FD causes considerable burden on the affected patient and society[12].There is now evidence to support the fact that functional disorders such as FD impair the affected patients to a similar extent as a disease with well-defined structural lesions; this disorder also causes considerable loss of working time and a large financial expense for medical management[13].

There is substantial evidence to suggest that patients with FD have a higher incidence of psychological disorders than population controls[14]. Patients with unexplained gastrointestinal complaints are more likely to suffer from symptoms of neurosis, anxiety, hypochondriasis and depression than controls[15,16]. Some studies have also revealed that there are possible links between emotional factors and changes in gastrointestinal physiology which may lead to abnormal gastric secretion, gut motility and function[17].

Life stress contributes to symptom onset and exacerbation in the majority of patients with FD[18]. Acute stress affects upper gastrointestinal motility and gastric acid secretion. However, studies of acute stress in FD patients have conflicting results[19]. A longitudinal study assessed the relationship of chronic and severe life stress to subsequent symptom intensity in patients with FD. The results of this study showed that severe and chronic threat has large and consistent effects on symptom intensity over time in FD patients[18].

VISCERAL HYPERSENSITIVITY

Visceral hypersensitivity is currently regarded as the mechanism responsible for both motor alterations and abdominal pain in functional bowel disorders including FD[20]. In order to elucidate the origins of visceral hypersensitivity it is necessary to understand the neuroanatomy and physiology of visceral sensation itself. Enteroendocrine cells in the lining of the gut act as chemical and mechanical transducers for local reflexes of initiation of afferent projections to the CNS[21]. Gut afferent signals reach conscious perception through a three neuron chain[4]. The cell body of the first order neuron is in the dorsal root ganglion and this neuron terminates in the dorsal column of the spinal cord. En passant fibres project to noradrenergic neuron in the prevertebral ganglia. This reflex centre results in modulation of viscus functions such as motility. Somatic and visceral afferents converge on dorsal horn neurons and result in viscerosomatic projection or referred pain. Descending controlling fibres such as serotonergic and noradrenergic from brainstem centres alter the sensitivity of the dorsal horn neurons and therefore centrally control the intensity of perception during visceral stimulation[21].

The second order neuron projects from the dorsal horn of the spinal cord to the thalamus and reticular formation in the brainstem. The ascending pathways are found in the spinoreticular and spinothalamic tracts. Nociceptive spinal pathways have recently been identified in the dorsal column of primates. These project nociception from viscera such as pancreas, colorectum, and duodendum (the upper bowel)[22]. This was also found in studies using rats[23]. These second order neurons synapse with satiety and autonomic centres and also the third order neurons that lead to emotional responses (limbic system) and conscious perception (sensory cortex). These projections lead to alterations in pulse rate, blood pressure, appetite and emotions in response to visceral pain. The loci of projection in the sensory cortex are not fully elucidated yet[24]. At present there is a limited understanding of the cerebral processing of visceral stimuli, the pathways and mediators of visceral afferents, the role of end organ modulation of sensation and association of symptoms with sensorimotor dysfunctions. Among the neurotransmitters involved in visceral perception are serotonin and noradrenaline. Different receptor subtypes for the transmitters may also have differential effects[24].

NEUROANATOMY OF THE SEROTONIN AND NORADRENALINE SYSTEMS

Serotonin is a neurotransmitter in the CNS and in the gastrointestinal tract. There are seven known serotonergic receptors, of which 5-HT_1, 5-HT_3, 5-HT_4 receptors (and their subtypes) seem to play an important role in the gut[25]. Centrally serotonin neurons are found at most levels of the brainstem and are concentrated in the raphe nuclei. Serotonergic neurons innervate virtually all parts of the CNS. Projections from the rostral raphe nuclei reach the forebrain, providing a cortical innervation that is the densest in sensory and limbic areas. Caudal raphe nuclei provide most of the projections to the brainstem and spinal cord.

Noradrenaline systems are also located in the CNS and within the gastrointestinal tract. There are two main types of adrenoreceptors α and β. There is two of each of the receptors: α_1 and β_1 are located on the post-synaptic membrane while α_2 and β_2 are found on the pre-synaptic membrane. Noradrenergic neurons are found in the pons and the medulla of the brainstem. Most are located in an area called the locus ceruleus, a collection of pigmented cells located near the floor of the fourth ventricle. The remainder of noradrenergic neurons are found in the lateral parts of the medullary reticular formation, in some nuclei associated with cranial nerves (the solitary nucleus and the dorsal motor nucleus of the vagus). Collectively these neurons innervate nearly the entire CNS. Ascending fibres reach the thalamus, hypothalamus, limbic forebrain structures and the cerebral cortex. All areas of the cerebral cortex receive some noradrenergic innervation with

the somatosensory cortex receiving a particularly dense innervation. Descending fibres project to other parts of the brainstem and to all spinal levels as well as the cerebellum[26].

INTERACTION OF SEROTONIN AND NORADRENALINE SYSTEMS

Central serotonergic pathways have been implicated in the mechanisms of nociception. Conflicting results have been published because serotonin administered intrathecally is seen to either inhibit or stimulate nociceptive responses. This may depend on the dose and the species studied[20].

While both supraspinally and spinally projecting serotonin pathways are involved in the modulation of nociceptive transmission, it is the pathways originating in the brainstem and projecting to the dorsal horn of the spinal cord that have been studied the most. This is due to the proposal of an endogenous pain-suppressing system using serotonin[27]. The actions of serotonin depend on the receptor subtype being activated and the response may be pronociceptive or antinociceptive[28]. Activation of spinal 5-HT_{1B} and 5-HT_3 produces an antinociceptive effect while activation of 5-HT_{1A} receptors has a pronociceptive effect. Activation of 5-HT_2 receptors produces antinociception which may be preceded by pronociceptive responses[29]. Serotonin interacts with other brainstem projection pathways including noradrenaline[28].

Studies were undertaken by Sawynok *et al* (1996), in order to investigate the interactions between serotonergic and noradrenergic systems in the modulation of pain in the rat. They provide evidence that the spinal antinociceptive effects of serotonin are dependent on noradrenaline. This is true as the antinociceptive effect of intrathecal (it) serotonin is reduced by depletion of noradrenaline in the spinal cord after the administration of the neurotoxin 6-hydroxydopamine[30]. The nature of the serotonin-noradrenaline interaction was investigated using pharmacological agents that interact with adrenergic mechanisms in different ways. Serotonin agonists produced a dose-related antinociception. This action was blocked by the administration of a non-selective antagonist for noradrenlaine and an antagonist selective for α_2 receptors but not by the α_1 and α_{2a} antagonist. But this observation is not definitive as α adrenoceptor antagonists could block the action of serotonin and related agonists by a number of mechanisms. Such mechanisms may include serotonin causing the release of endogenous noradrenaline by perhaps a receptor mediated reaction[31]. Serotonin could also interact synergistically with endogenous noradrenaline. The adrenergic system is tonically active within the spinal cord[32] and serotonin may depend on such tonic activity for expression.

In order to determine which serotonin receptors were involved in the interaction a number of serotonin agonists each selective for a different serotonin receptor were administered to rats. Pre-treatment with 6-OHDA produced a marked depletion in noradrenaline levels in the spinal cord (>80%) and reduced antinociception by serotonin and by most of the 5-HT_1 agonists but not the 5-HT_3 or 5-HT_2[28]. The agents that were inhibited by 6-OHDA were potentiated by a systemic pre-treatment of desipramine (blocks the reuptake of noradrenaline). This data presents several possibilities: (1) serotonin releases endogenous noradrenaline from the spinal cord through a receptor mediated action. This indicates that the action of serotonin is augmented by desipramine. This is consistent with a serotonin receptor mediated release of noradrenaline and augmentation resulting from a blockage of the reuptake of noradrenaline. (2) The serotonin receptor involved in this interaction appears to be a 5-HT_1-like receptor[28] as depletion of noradrenaline reduced the antinociception of the 5-HT_1 agonists and not others.

ACTION OF CENTRAL NORADRENALINE AND SEROTONIN RECEPTORS ON GASTROINTESTINAL MOTILITY

Gastrointestinal motility and secretions are modulated by cerebral nuclei via autonomic efferents in a coordinated manner. The sympathetic division of the ANS is important in the control of gastrointestinal function both in the basal and under stressful conditions[33]. Noradrenergic α_2 receptors in the hypothalamus form an important part of this network and research has demonstrated that these receptors have a significant influence on intestinal motility and transit time.

In several species, including humans, the sympathetic nervous system exerts a primarily α_2 mediated tonic inhibitory effect on gastrointestinal motor function[33]. α_1 and β_2 agonists also reduce colonic contractile frequency in non-human primates[34].

Noradrenaline (10 nmol) administered intracisternally (i.c.) significantly decreased gastric motility while yohimbine and not prazosin abolished this decrease pointing towards the involvement of the α_2 receptor in noradrenergic regulation of gastric motility[35]. The α_2 agonist clonidine acting centrally produces a dose dependent decrease in intestinal motility[36]. These studies indicate that central noradrenergic receptors play a role in the modulation of gastrointestinal motility and that alterations in this system may lead to motility symptoms seen in FD patients.

Peripheral serotonin and its receptors effects on gastrointestinal motility have been well established but the control of central serotonergic receptors has been somewhat overshadowed. This may be due to the fact that the majority of serotonin is present in the gut and only 5% exists in the CNS. Some treatment studies suggest that centrally acting serotonergic agents alleviate motility symptoms in FD patients. But it is not clear whether this is due to central or peripheral effects of these drugs. Serotonin is present in enterochromaffin cells (EC) and enteric neurons in the gastrointestinal tract. Release of serotonin from EC cells act as chemical and mechanical transducers for the initiation of local reflexes (peristalsis) and for activation of projections to the CNS[37].

ALTERATIONS IN VISCERAL SENSATION AND POSSIBLE CAUSES

Patients with FD have an increased perception of physiological or minor noxious stimuli in both the fasting and postprandial states[38]. When compared to normal healthy controls, FD patients are hypersensitive to isobaric or isovolumetric balloon distension of the proximal stomach. In view of normal gastric wall compliance in these studies, increased sensation during gastric distension suggests abnormal afferent function or the "irritable stomach syndrome"[39]. This enhanced perception of visceral stimuli may be due to greater sensitivity of visceral afferent pathways or a central amplification of visceral afferent input[40]. Visceral afferent input is modulated by a variety of mechanisms operating between the gastrointestinal tract and the CNS, and dysfunction of these regulatory systems could distort gastrointestinal perception[41]. The ANS which regulates gastrointestinal function is thought to modulate visceral sensitivity. Increased sympathetic tone is known to increase the level of perception of gastrointestinal stimuli without affecting the reflex responses[42]. Alterations in sympathetic modulation of visceral sensitivity may therefore play an important role in FD[41]. Since noradrenaline is the principal neurotransmitter and receptor system involved in the sympathetic nervous system, it may be fair to speculate that there is an abnormality in this system in FD patients. This may be present as an alteration in sensitivity or regulation of the noradrenergic receptor system.

Hypersensitivity of the dorsal horn neurons or altered supraspinal processing of visceral afferent function has been implicated in the increase in visceral sensation[5]. This hypersensitivity may develop in response to descending influences from the brainstem. With regard to the clinical characteristics and the concept of central hypersensivity, a model has been proposed by which abdominal pain in FD could develop by multiple mechanisms either alone or in combination[21]. Stress is involved in the exacerbation of symptoms in FD. Stress induced analgesia is modulated by descending pain inhibitory pathways that are partially mediated by serotonergic systems. This stress induced somatic hypoalgesia can be accompanied by a stress induced visceral hyperalgesia[43]. This may be due to both pain facilitatory and inhibitory systems being activated simultaneously by stress, with the net effect being determined by the contribution of each system[44].

If there is a dysregulation of the descending pain inhibiting serotonergic system, then this would at least partially explain the visceral hyperalgesia of FD patients. Stress is associated with the onset and exacerbation of symptoms in these patients. It may provoke an increase in visceral hyperalgesia in individuals who are vulnerable due to the inability to inhibit the pain facilitatory mechanisms due to a dysregulation of their central serotonergic systems.

ALTERATION IN MOTILITY AND POSSIBLE CAUSES

FD is associated with gastrointestinal motor abnormalities such as delayed emptying[45], impaired initial distribution of a meal within the stomach[46], impaired accommodation[38] antral hypomotility, gastric dysrhythmias (tachygastrias, bradygastrias and mixed dysrhythmias) and altered duodenojejunal motility[5]. The occurrence of dyspeptic symptoms after food digestion suggests a disturbance of postprandial gastric motility which leads to slowed gastric emptying accompanied by sensations of prolonged gastric distension, bloating, and nausea. It was Coffin *et al*[47] that initially suggested that patients with FD may have abnormal motor function of the proximal stomach. Usually, after a meal the proximal stomach relaxes in order to act as a reservoir and to enable an increase in gastric volume without a significant increase in gastric pressure. This impaired accommodation is a disturbance of the "diastolic" function of the stomach and is a frequent finding in FD patients[5].

The underlying mechanism for this impairment of gastric motor is not fully understood. But it has been proposed that there may be a dysfunction of the ANS[48]. Noradrenergic modulation of synaptic vagal transmission in the nucleus tractus solitarus can be assumed to play a role in the modulation of vagovagal reflexes. These include regulation of gastric accommodation, regulation of spontaneous transient lower oesophageal sphincter relaxation and duodenogastric reflexes[40]. Therefore, alterations in this noradrenergic receptor system can lead to abnormalities of motor function and accommodation.

As mentioned, stress plays a role in the onset and exacerbation of symptoms in functional gastrotintestinal disorders such as FD and chronic stress leads to overactivity of these systems. A downregulation of autoreceptors (α_2 and 5-HT_{1A} receptors) was observed in an animal model of social stress[49] and in patients with posttraumatic stress disorder[50] which results in an enhanced release of noradrenaline and serotonin respectively. This enhanced release may lead to a downregulation of postsynaptic receptors (such as β adrenergic and α_1 receptors)[49]. These neuroplastic alterations would initially increase noradrenaline release (presynaptic) but ultimately decrease postsynaptic target neurons. Also excessive release of transmitters could result in depletion of serotonin or noradrenaline further decreasing postsynaptic neuron activation[40].

Stress among other factors can lead to alterations in central aminergic networks involving serotonin and noradrenaline. These alterations may result in changes in the functioning of the ANS and therefore may have an impact on gastrointestinal functions such as motility.

TREATMENT OF FD

Although treatment of FD is expensive, the agents are rarely used in a systematic manner: the majority of treatments are empirical and the results are short lived once the therapy ends. To a degree, this is due to lack of consistent pathophysiologic markers which is why treatment of FD is symptom driven. Eradicating *Helicobacter pylori*, if present, is a first-line strategy. Patients with upper abdominal pain (ulcer-like) as the predominant symptom can initially be treated with proton

pump inhibitors. Those that suffer from dysmotility-like symptoms can be treated with acid suppressive therapy, prokinetic agents or 5-HT_1 agonists. The use of prokinetics is limited due to availability issues[51]. Some studies suggest that 5-HT_1 agonists are effective in the treatment of impaired accommodation[2].

Many patients with FD also suffer from psychiatric disorders including anxiety, mood and somatoform disorders[52] so that the expanding field of psychologic therapies provide a promising area of treatment. Psychoactive agents such as antidepressants and anxiolytics with serotonergic activity have been widely used in the treatment of patients with FD. But it is unclear if these medications improve symptoms through central or peripheral mechanisms[53].

Nonpharmacological interventions have also been employed with two studies having reported a favourable outcome with psychotherapy in FD[53,54]. A Cochrane review on psychological interventions (such as psychotherapy, cognitive behavioural therapy and hypnosis) in FD patients reported beneficial results of such therapy[3].

STUDIES

At present, an integrated disease model that adequately explains the broad variety of symptoms seen in FD, as well as the association with extra-intestinal syndromes and psychiatric disorders is lacking. A number of receptors may be involved in symptom production in FD patients. One study suggests that there is an increased sensitivity of central serotonin receptors in these patients[55]. The authors examined the functional activity of central serotonin by means of a neuroendocrine challenge. The release of the hormone prolactin is partially under serotonin control. The anxiolytic buspirone was used to stimulate the 5-HT_{1A} and increase prolactin release. Although not entirely specific for serotonin receptors (it also acts on dopamine receptors) there is enough evidence to indicate that its mediation of prolactin release is via serotonin receptor as this action can be blocked with the serotonin antagonist methysergide[56]. Dopamine responses in FD were examined using bromocryptine as a probe drug and prolactin decrease as the response. The response of the FD patients was not any different from that of the controls. So it was concluded that buspirone provided an acceptable method of serotonin-stimulated prolactin release. When FD patient were administered the dose of buspirone the response was 200% greater than in healthy controls. This indicates that patients with FD have a supersensitivity of central 5-HT_{1A}[55].

FD is sometimes referred to as upper irritable bowel syndrome (IBS) due to the overlap of symptoms[57]. A down regulation in the noradrenergic α_2 receptors has been implicated in IBS[58]. This may apply to a subgroup of FD patients. The function of these receptors was assessed using a neuroendocrine test. The test was based on the fact that the monoamine reuptake inhibitor desipramine acts on central α_2 receptors to bring about the release of growth hormone[59]. This effect is blocked by the α_1 and α_2 receptor antagonist phentolamine but not by the α_1 antagonist prazosin[60]. IBS patients displayed blunted growth hormone response to a challenge with desipramine at 1 mg/kg body weight. Similar blunted growth hormone responses are seen in depressed patients[61] but this does not account for the blunting here. All patients in this study were assessed by a consultant psychiatrist and while five out of the total 13 were diagnosed with depression both the depressed and the non depressed patients displayed blunted responses[58]. Also it may be argued that delayed gastric emptying and slowed absorption of desipramine might explain the findings. But this was not the case as no further elevations in growth hormone were noted in samples taken later. These results are due to the central action of the α_2 receptors. These findings indicate that the abnormal functioning of these receptors may be important in IBS and a subset of FD and may offer new treatment options[58].

A dose response study using nitroglycerin and clonidine demonstrated that gastric relaxation and antinociception have different effects on the perception of visceral stimuli in humans[38]. Both of these drugs caused relaxation of the fasting stomach without impeding normal postpradial accommodation of the proximal stomach. The gastric balloon distension test was used to measure the antinociceptive effects. Perception scores were reduced with clonidine but not with nitroglycerin. The nitroglycerin actually increased bloating despite significant relaxation. This data suggests that relaxation alone is insufficient to reduce sensation in response to distension in humans[38]. This study suggests that central α_2 receptors are involved in the perception of visceral stimuli and that activation of these receptors produces an analgesic effect in FD patients. This suggests that these patients may have some abnormality of the α_2 receptors that may influence the hypersensitivity seen in FD patients.

In summary the main symptoms of FD are alterations in motor function and abdominal pain. The mechanisms involved in the modulation of these functions include central serotonergic and noradrenergic receptor systems. Both human and animal studies indicate that there are abnormalities in these systems in FD which may account for the symptoms of FD.

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TOPIC HIGHLIGHT

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Cholecystokinin hyperresponsiveness in functional dyspepsia

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Abstract

Functional dyspepsia (FD) is a common disorder of yet uncertain etiology. Dyspeptic symptoms are usually meal related and suggest an association to gastrointestinal (GI) sensorimotor dysfunction. Cholecystokinin (CCK) is an established brain-gut peptide that plays an important regulatory role in gastrointestinal function. It inhibits gastric motility and emptying via a capsaicin sensitive vagal pathway. The effects on emptying are via its action on the proximal stomach and pylorus. CCK is also involved in the regulation of food intake. It is released in the gut in response to a meal and acts via vagal afferents to induce satiety. Furthermore CCK has also been shown to be involved in the pathogenesis of panic disorder, anxiety and pain. Other neurotransmitters such as serotonin and noradrenaline may be implicated with CCK in the coordination of GI activity. In addition, intravenous administration of CCK has been observed to reproduce the symptoms in FD and this effect can be blocked both by atropine and loxiglumide (CCK-A antagonist). It is possible that an altered response to CCK may be responsible for the commonly observed gastric sensorimotor dysfunction, which may then be associated with the genesis of dyspeptic symptoms.

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Key words: Functional dyspepsia; Cholecystokinin hyperresponsivenes; Stress; Sensorimotor dysfunction

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INTRODUCTION

Functional dyspepsia (FD) is a heterogeneous condition of uncertain etiology. It is a common disorder frequently encountered, both in the primary care and specialist clinics. FD in the current Rome II criteria refers to, persistent upper abdominal pain or discomfort with or without symptoms of fullness, bloating, early satiety and nausea, with no identifiable organic cause. Excluded are patients with reflux symptoms and lower gastrointestinal (GI) symptoms. It also stipulates that symptoms must be present for at least 12 wk which need not be consecutive, in the preceding 12 mo[1]. The symptoms patients complain of are variable, occasionally very disabling and associated with a reduced quality of life, which is determined by symptom severity. The dyspeptic symptoms are usually meal related, however the temporal relationship between dyspeptic symptoms and meal intake need to be studied further. Symptoms are usually intermittent and can be aggravated by food[2] and stress[3]. Depending on the symptom clusters, patients may be categorized into different subgroups. Dysmotility-like dyspepsia describes a group that, on symptoms alone, appears to have GI sensorimotor dysfunction. The other two subgroups include Ulcer-like dyspepsia and mixed group. Studies have implicated among others GI dysmotility, gastric hypersensitivity, abnormality of accommodation and an altered brain-gut communication as important pathophysiological correlates of FD. It can be thought of as a biopsychosocial disorder, in that there is an underlying GI sensorimotor dysfunction with an important input from the psyche[4].

Cholecystokinin (CCK) is an established brain-gut peptide that plays an important regulatory role in gastrointestinal function. CCK is involved in the control of food intake in both man and animals[5-7]. It inhibits gastric motility and emptying via capsaicin-sensitive vagal pathways in rats[8]. In human studies, CCK mediates gastric emptying under physiological conditions[9]. It also inhibits food intake and increase satiety in humans[10]. CCK appears to be involved in the gastric response to the presence of several intestinal stimuli, including fat and protein. In humans, CCK pathways modulate sensations induced by gastric distension[11]. It has also been hypothesized that CCK plays an etiologic factor in panic disorder and anxiety[12], factors that may be important in precipitating or aggravating symptoms in FD patients. CCK plays an important role in the brain-gut axis, allowing peripheral signals from the gut to modulate central pathways and vice-versa. An altered CCK function in terms of response to food intake, abnormal central response to stressors or

even disturbed effect on gut sensorimotor functions may be responsible for the dyspeptic symptoms in FD and involved in the pathophysiology of this common disorder.

SYMPTOMATOLOGY IN FD

The correlation between symptoms and proposed impaired GI function is still unclear. Stanghellini investigated the relationship between symptom severity, demographic features and gastric dysmotility in a large series of FD patients[13]. They were able to identify two main subgroups, the first has predominant epigastric pain, male gender and normal gastric emptying, while the second group is characterized by predominantly nonpainful symptoms, female gender, a high association with irritable bowel syndrome (IBS) and delayed gastric emptying. The same group also reported that postprandial fullness and severe vomiting were independently associated with delayed gastric emptying of solids. These findings were confirmed by Sarnelli *et al* who studied the relationship between FD and delayed gastric emptying of solids or liquids and discovered that delayed gastric emptying of solids was constantly associated with postprandial fullness and vomiting. While delayed emptying of liquids was associated with early satiety and postprandial fullness[14]. However the above findings were disputed by Talley *et al* who found no predictive value using specific symptoms to identify alterations of gastric emptying[15].

Studies have suggested that in FD, gastric accommodation is normal during fasting but is impaired after a meal, due to failure of proximal stomach relaxation[16,17]. Other reports suggested that impaired accommodation is associated with symptoms of early satiety and weight loss. Furthermore restoring gastric accommodation with fundus relaxing drugs, 5HT1 agonists sumatriptan and buspirone[18], Tack *et al* were able to improve the symptom of early satiety. The above findings however, were contradicted by Boeckxstaens *et al* who could demonstrate no difference between postprandial symptoms and proximal stomach function[19]. Hypersensitivity to gastric distension has previously been reported in FD[20], but it is present only in a subset of patients. The relevance of this particular characteristic observation was studied by a group from Belgium[21] who discovered that gastric hypersensitivity is associated with symptoms of postprandial epigastric pain, belching and weight loss. Similar observations were reported by Salet *et al* who reported that gastric visceral hypersensitivity plays an important role in the pathogenesis of upper gastrointestinal symptoms especially nausea, abdominal bloating and pain[22].

More importantly FD patients were known to report symptoms after a meal, including fullness, bloating, epigastric pain, nausea and vomiting. Based on symptoms alone, this seems to suggest an underlying gastric sensorimotor disorder. However the temporal relationship between symptoms, meal ingestion and gastric dysmotility has not been truly examined. Ingestion of a fatty meal in FD patients will result in the induction of dyspeptic symptoms[2]. The presence of nutrients in the small intestine slows gastric emptying and suppresses appetite and food intake which is mediated in part by CCK[23,24]. CCK has also been implicated in the induction of nausea in healthy subjects[25,26]. It relaxes the proximal stomach[27] and delays gastric emptying. Thus CCK may play an important role in symptoms production in patients with FD.

CCK AND GI HYPERSENSITIVITY

Prevalence of hypersensitivity to gastric distension in FD has previously been reported[20,28,29]. Gastric distension is perceived as painful at smaller distending volumes and lower pressures compared to a group of healthy controls. The observed hypersensitivity to gastric distension is associated with epigastric pain, belching and weight loss. There is also evidence of sensitivity to other stimuli including acid and digestive products of fat and protein. Furthermore we have previously demonstrated that FD patients responded differently to an infusion of CCK octapeptide (CCK-8), compared to a group of healthy and disease controls[46].

Response to CCK-8 was assessed by an intravenous CCK-8 infusion (6 ng/kg per minute) in a double-blind, cross-over fashion using normal saline solution as placebo infusion. The test was deemed positive when the infusion reproduced part or all of the patients' symptoms. The severity of the response was evaluated on a simplified visual analog scale (VAS). The VAS allowed us a reproducible method of assessing symptoms and a crude form of grading patients' responses. It also provided a means of analyzing severity of symptoms and allows us to grade individual's responses to the CCK-8 infusion. Furthermore we can also determine the efficacy of atropine and loxiglumide in alleviating symptoms.

The most common reproducible symptom in the CCK challenge test is that of upper abdominal pain. This is usually associated with a feeling of abdominal bloating and satiety. Other symptoms included borborygmia, nausea, vomiting, light-headedness and occasional heartburn. The test is negative if the infusion fails to precipitate any symptoms or only an occasional mild discomfort. In some cases the precipitated symptoms were so severe that the infusion had to be discontinued due to patients' distress. Majority of FD patients had a positive response to the CCK-8 challenge. In all the responders, the symptoms produced were quite marked and there was little doubt about the diagnosis. None of the healthy controls reported any significant symptoms to the CCK-8 infusion. The few that experienced minor epigastric discomfort or nausea can be explained by the physiological action of CCK. None of the subjects reported any sensation from the saline infusion.

It was also demonstrated that the response to CCK-8 can be blocked by atropine in a dose dependent fashion. Atropine also managed to prevent recurrence of symptoms to further CCK-8 infusion. The CCK-8 challenge test, likewise can be attenuated using the CCK-A antagonist loxiglumide.

Scintigraphic gastric emptying was also determined in the FD group of patients using radiolabelled technetium and solid phase gastric emptying was shown to be significantly delayed in this group of patients compared to

a group of healthy controls.

Katchinski demonstrated that sham feeding in healthy volunteers brought about a stimulation of gastrointestinal motility implying a cephalic stimulation of GI motility. He also showed that the peripheral acting cholinergic antagonist atropine completely abolished antral activity and consequently coordinated antroduodenal motor activity, while Loxiglumide inhibits cephalic stimulation of antral contractions[30]. Furthermore, atropine completely blocked the changes in gastric function induced by stimulation of the nucleus raphe obscurus[31]. Similarly, the dyspeptic symptoms produced by the CCK infusion were completely abolish by atropine, indicating the importance of the vagal efferent pathways in mediating this response. The suppression of response by atropine may occur also at the level of the peripheral muscarinic receptors, at the junction of the gastric musculature or the secretory glands.

Hypersensitivity to CCK may explain the abnormal response to CCK infusion in dysmotility-type FD patients. The exaggerated response to CCK in this group of patients may be the basis of the pathophysiology of the condition and also account for the observed dyspeptic symptoms. Exogenous CCK may act on primary afferent neurons that are also gastric mechanoreceptors and there follows activation of a vago-vagal reflex pathway, which leads to relaxation of the body of the stomach[8,32-34]. Early satiety and post-prandial fullness may be mediated via the afferent sensory pathways or through central CCK interconnections. Meanwhile, abdominal discomfort, pain, distension, nausea and vomiting may result from excessive fundal relaxation mediated via the motor limb of the vago-vagal reflex, activated by CCK, gastric distension or possibly a combination of both. This reflex results in the inhibition of gastric emptying which is a predominant feature of FD. Any abnormalities which up-regulate sensory signals anywhere within the central nervous system and enteral nervous system (CNS-ENS), could induce hypersensitivity leading to pain and discomfort which characterise FD. Over stimulation of the afferent limb of the multiple sensorimotor reflexes anywhere from the GI tract to the CNS could trigger a motor response. Depending on the pathway stimulated, a different response will be obtained.

CCK CHALLENGE TEST AS A DIAGNOSTIC TEST

FD is a diagnosis of exclusion, though a positive diagnosis can be made based on the symptoms[1], efforts should be made to exclude organic diseases if there were presence of "alarm signs or symptoms". The CCK-8 challenge test may be used as a diagnostic tool to positively identify dysmotility type FD. For it to be effective the challenge test should be safe, able to reproduce patients symptoms, response should be reproducible and dose dependent and blocked by the antagonist and most importantly specific to the disorder. We have shown that the test is safe and can precipitate the patients' symptoms which can be blocked by Atropine and Loxiglumide (CCK antagonist). The CCK-8 challenge test is dose dependent, in that no response was seen with 2 ng/kg per minute, moderate response seen with 4 ng/kg per minute and best response identified with the 6 ng/kg per minute. The test is reproducible in that in 10 patients when the test was repeated on a separate day, there was no significant difference in the reported symptom severity (on the VAS), score of 35 (5) *vs* 36 (7) [mean (SD)]. However the test needs to be tested further in other functional disorders especially in Irritable Bowel Syndrome (IBS) patients or patients with Non-erosive reflux disease. Thus more work need to be done before the CCK-8 challenge test can be reliably used as a diagnostic test.

FOOD SENSITIVITY AND CCK IN FD

CCK is released by the duodenum in the presence of digestive products of fats and proteins in the intestine[35]. We have previously seen that physiological CCK relaxes the stomach, constricts the pylorus[36,37] and inhibits gastric emptying,[38] and thereby increases gastric distension. This effect of CCK is mediated via gastric vagal afferents[10]. Furthermore in healthy subjects, the combination of duodenal lipid and gastric distension induces meal-like fullness and nausea. Duodenal lipid reduces gastric tonic and phasic activity during distension, and this can be partially block by loxiglumide[39]. This signifies that CCK-A receptors are implicated in the cause of nausea and postprandial fullness, associated with duodenal lipid infusion. The increased gastric distension induced by the delayed gastric emptying may be the method by which CCK reduces food intake[5,38].

Similarly in FD the presence of certain nutrients especially fat, in the intestine can generate dyspeptic symptoms, including nausea, bloating, pain and fullness[40-43]. Feinle *et al* demonstrated that in the FD patients, duodenal infusion of lipid aggravates the hypersensitivity to gastric distension. This effect is specific for fat, and evidence suggests the participation of CCK[44]. The fat induced dyspeptic symptoms can also be abolished by dexloxiglumide (CCK-A antagonist) signifying an involvement of CCK-A receptors[42]. The underlying mechanism is probably due to hypersensitivity to CCK. We postulate that it is CCK that enhances the distension effect, rather than distension enhancing the effects of CCK. Furthermore recordings from the afferent vagal nerve indicates that mechanoreceptors sensitive to gastric stretch respond more strongly in the presence of CCK[45].

Endogenous CCK released in response to lipid infusion acts in a paracrine fashion to stimulate CCK-A receptors. The information is then transmitted to the CNS via vagal afferent nerves. This pathway is involved in the feedback inhibition of gastric tone and motility which could indirectly gives rise to the observed symptoms[42,46]. However CCK may not be the only factor involved in the above vagal reflex pathway. It is more common indigestion, trying to eat when we are emotionally stressed[5]. Psychological and physical stress has been shown to cause slowing of gastric emptying in animals. Furthermore, excitation of the sympathetic nervous system by stressors increases visceral sensitivity[47,48].

ROLE OF CENTRAL SEROTONERGIC AND NORADRENERGIC RECEPTORS

Stress also plays an important role in precipitating or aggravating symptoms in FD. Central serotoninergic (5HT) receptors are important in mediating the stress response. Utilizing the Buspirone Neuroendocrine challenge test, we have previously shown that FD patients have hypersensitive central 5HT receptors functioning[49]. The release of prolactin from the anterior pituitary is under the inhibitory control of dopamine and stimulatory control of 5HT. Buspirone, an azaspirodecanedione, stimulates central 5HT1A receptors and causes prolactin release in a dose dependent manner, and the extent of prolactin release post stimulation can be used as an index of central 5HT receptors function. Prolactin release following Buspirone challenge was reported to be significantly greater in FD patients. 5HT plays an important role in the control of GI function both peripherally at the ENS and also centrally at the CNS. Similarly, we have also shown that the observed hypersensitivity of the central 5HT receptors were highly correlated with delayed gastric emptying[50].

Central noradrenergic pathways are also important in the mediation of the stress response. α-2 receptor activation mediates inhibition of a number of gastrointestinal functions including intestinal motility. Its activation also inhibits acetylcholine release from the vagal nerve. Previously we have shown that the central α-2 receptors are hyposensitive in patients with delayed gastric emptying[51] using the desipramine neuroendocrine challenge test. In this test, subjects were challenge with desipramine, a monoamine reuptake inhibitor, which blocks the reuptake of noradrenaline when given orally. This indirectly stimulates growth hormone release by acting on central α-2 receptors situated in the hypothalamus.

In fact FD patients who have an abnormal response to the CCK-8 challenge test, have been shown to have hypersensitive central 5HT receptors (as evidenced by an exaggerated prolactin response to buspirone challenge) and hyposensitive central α-2 or noradrenergic receptors (as evidenced by a blunted growth hormone response to Desipramine challenge). Gastric emptying was also shown to be delayed in this group of patients[52]. Kendrick established that injections of CCK stimulated significant increase of noradrenaline and serotonin in the paraventricular and supraoptic nucleus. Both hypothalamic nuclei receive noradrenergic and serotonergic inputs and are important in the regulation of feeding and control of GI motility. The principal action of systemically administered CCK centrally is via the nucleus tractus solitarius (NTS) which projects directly or indirectly to the paraventricular nucleus (PVN) and supraoptic nucleus (SON) and the dorsal motor nucleus of the vagus (DMV). In the PVN, systemic injections of CCK activate neurons which influence feeding behaviour and affect gastric motility[7,33].

CCK AND PSYCHOLOGICAL STRESS

Both depression and chronic tension can present with abdominal symptoms[53,54]. Studies have shown that patients with FD have increased levels of anxiety. Gomez and Daly reported that 22% of their FD patients had chronic tension and a further 15% demonstrated hysterical symptoms[55]. Mangi *et al* reported that two thirds of 30 consecutive outpatients with FD met DSM-III criteria for anxiety disorders[56]. The neurotransmitter CCK can be found in abundance in the brain. Two receptor subtypes (A and B) have been identified within the CNS. CCK-B receptors are more numerous and widely distributed in the CNS[57-59]. CCK or CCK receptors are discovered in areas of the brain involved with cognitive or emotional aspects of behaviour[58,60]. It is known to interacts with other neurotransmitters in the CNS (monoaminergic, serotonergic, GABAergic) known to be involved in anxiety and affective disorders[61-63]. Chronic treatment with imipramine, which inhibits noradrenaline and 5HT reuptake, could antagonize the panic causing effects of CCK-4 in panic disorder patients, indicating that these monoamines may be important in interacting with CCK in promoting panic symptoms. Thus CCK-B receptor plays an important role in anxiety states and panic attacks[12,64,65]. CCK-B receptors can modulate activity within the hypothalamic-pituitary-adrenal (HPA) axis[65,66], which is involved in the stress response.

Psychological stress is widely believed to precipitate exacerbation of symptoms in FD. Inhibition of gastric emptying and stimulation of colonic transit is the most consistent motility response of the GI tract to acute stress. An organism's response to stress is generated by a complex integration of neurons in the CNS. Main output of this integrated central network to the periphery (which is activated in response to various stressors, both physical and psychosocial) includes autonomic and neuroendocrine responses together with modulation of pain. Present evidence strongly suggests that acute stress affects visceral sensitivity in humans. Whether chronic stress plays the same role remain to be seen. Early life stress and trauma, in the form of abuse, neglect, or loss, play a major role in the vulnerability of individuals to develop functional GI disorders. It is proposed that in the genetically predisposed individual, both early life stress and severe life-threatening stress can result in permanent alteration in central stress circuitry and predisposes the individual to the development of functional disorders later in life.

LOXIGLUMIDE IN THE TREATMENT OF FD

If CCK plays an important role in the pathophyphysiology of FD then the use of Loxiglumide (CCK-A receptor antagonist) should be effective in the control of the symptoms. A randomized, double-blind, parallel group, placebo-controlled trial was conducted in 28 symptomatic FD patients (8 male, 20 female, aged 19-59 years, mean age 33). All patients satisfy the Rome II criteria for the diagnosis of FD. They all have normal endoscopy, normal abdominal ultrasonography, a positive CCK provocation test (symptom appearance after CCK-8 6 ng/kg iv infusion) and delayed (half emptying time > 60 min for the solid phase) gastric emptying of a radiolabeled, mixed solid-liquid meal of about 400 Kcal. Patients were randomly assigned to placebo or

Loxiglumide 400 mg three times daily for 8 wk. Response to treatment was evaluated on a Visual Analogue Scale to generate a Cumulative Symptom Score (CSS). Despite an expected high rate of placebo response, a quicker, more pronounced, and sustained symptomatic relief was observed during treatment with loxiglumide. There were 11 of 12 responders on Loxiglumide compared to only 6 of 11 on placebo ($P = 0.04$ Chi-square test). Loxiglumide also showed an acceleration of solid phase gastric emptying whereas placebo did not show any effect. However there was no correlation observed between increased gastric emptying and clinical improvement. Thus Loxiglumide is effective in the treatment of symptoms of FD and has a prokinetic effect. However, we need further studies on a larger population of patients to confirm the findings[67].

In summary, the discomfort, pain and altered sensations (to intraluminal stress) that characterize FD are considered to represent visceral hyperalgesia. Mechanisms by which functionally disordered visceral receptors, respond abnormally to normal everyday stimuli remain unknown. However, it is also possible that an aberrant input to the CNS or altered CNS pathways (5HT, Noradrenergic and central CCK) could contribute to the observed visceral hyperalgesia and dysmotility. Hypersensitive visceral CCK receptors may be modulated by central pathways primed by stress or trauma. The receptors will then respond inappropriately to normal stimuli, activating efferent pathways via a vago-vagal reflex pathway to cause perturbation of peripheral sensorimotor functions. This may be the basis for the genesis of the symptoms encountered in FD.

Furthermore, the CCK-8 challenge test may also be a useful tool for the diagnosis of Dysmotility type FD, but this will need further evaluation.

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TOPIC HIGHLIGHT

Andrew Seng Boon Chua, MD, *Series Editor*

Drug treatment of functional dyspepsia

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Abstract

Symptomatic improvement of patients with functional dyspepsia after drug therapy is often incomplete and obtained in not more than 60% of patients. This is likely because functional dyspepsia is a heterogeneous disease. Although great advance has been achieved with the consensus definitions of the Rome I and II criteria, there are still some aspects about the definition of functional dyspepsia that require clarification. The Rome criteria explicitly recognise that epigastric pain or discomfort must be the predominant complaint in patients labelled as suffering from functional dyspepsia. However, this strict definition can create problems in the daily primary care clinical practice, where the patient with functional dyspepsia presents with multiple symptoms. Before starting drug therapy it is recommended to provide the patient with an explanation of the disease process and reassurance. A thorough physical examination and judicious use of laboratory data and endoscopy are also indicated. In general, the approach to treat patients with functional dyspepsia based on their main symptom is practical and effective. Generally, patients should be treated with acid suppressive therapy using proton-pump inhibitors if the predominant symptoms are epigastric pain or gastroesophageal reflux symptoms. Although the role of *Helicobacter pylori* (*H pylori*) in functional dyspepsia continues to be a matter of debate, recent data indicate that there is modest but clear benefit of eradication of *H pylori* in patients with functional dyspepsia. In addition, *H pylori* is a gastric carcinogen and if found it should be eliminated. Although there are no specific diets for patients with FD, it may be helpful to guide the patients on healthy exercise and eating habits.

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Key words: Functional dyspepsia; Drug treatment; *Helicobacter pylori*; Predominant symptoms

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INTRODUCTION

Symptomatic improvement of patients with functional dyspepsia with current drug therapy is left with many unmet needs. This is because functional dyspepsia is a heterogeneous disease and a uniform response to drug treatment can therefore not be achieved. There are many possible factors to explain the difficulties achieving adequate symptom relief these patients[1-3]. The issues to be addressed when treating patients with functional dyspepsia: (1) Definition and classification of dyspepsia (Rome I, Rome II); (2) Associated upper and lower GI symptoms; (3) Predominant symptom (e.g. reflux, abdominal pain); (4) Number of symptoms; (5) Presence of alarm features (anemia, weight loss, dysphagia); (6) Co-morbidities; (7) Drug interactions; and (8) *Helicobacter pylori* (*H pylori*) status. One of the main factors that warrants clarification is the definition of dyspepsia. Although great advance has been achieved with the consensus definitions of the Rome I and II criteria, there are still some aspects about the definition of functional dyspepsia that require clarification. The Rome criteria explicitly recognise that epigastric pain or discomfort must be the predominant complaint in patients labelled as suffering from functional dyspepsia. However, this strict definition can create problems in the daily primary care clinical practice, where the patient with functional dyspepsia presents with many complex rather one simple symptom because on the average, patients with dyspepsia seen in primary-care settings report 5.5 abdominal symptoms, which also may vary over time[4,5]. In addition, patients with functional dyspepsia presenting to the primary care doctor are different than patients being evaluated at tertiary medical centres[1,2,6-9]. Before starting drug therapy it is recommended to provide the patient with an explanation of the disease process and reassurance. Ruling out an organic cause of their disease by endoscopy can lead to improved acceptance of the disease process. In general, the approach to treat patients based on their main symptom is practical and effective. The general approach is to treat patients with

acid suppressive therapy with proton pump inhibitors if the predominant symptom is epigastric pain or if there are gastroesophageal reflux symptoms present. The role of *H pylori* in functional dyspepsia continues to be a matter of debate. Nevertheless, recent data indicate that there is a modest but significant benefit in eradication of *H pylori* in functional dyspepsia. Although there are no specific diets for patients with FD, it may be helpful to guide the patients on healthy exercise and eating habits.

PROBLEMS WITH THERAPEUTIC TRIALS

The literature is full of reports on drug therapies for functional dyspepsia and the list of drugs used to treat functional dyspepsia is long[10-30] (Table 1). One of the most frustrating aspects about therapies in functional dyspepsia is that most medical treatments available to date have been shown to be of no or only limited efficacy. Furthermore the results of most of these studies do not apply to our current standards anymore. First, the definition of functional dyspepsia has changed over time. Second, most studies are difficult to interpret because of lack of rigorous design criteria, either because of small sample size, poor design, not blinded or not placebo-controlled. Smaller studies tend to show more efficacy than well-controlled larger ones. Abraham *et al* has demonstrated that the quality of trials has an impact on the efficacy estimates of treatment[31]. The authors performed a systematic review of randomized controlled trials of dyspepsia investigated using endoscopy from 1979 to 2003 using the Jadad score and Rome II guidelines[31]. They found that poor quality trials suggested a benefit of prokinetic therapy, which was not confirmed in high quality trials. Also there was a marked benefit of H2-receptor antagonist therapy in poor quality trials, but a marginal benefit in good quality trials. Two high quality trials suggested a limited benefit with the use of proton pump inhibitors, with no poor quality trials to provide a comparison[31] are (1) Setting where study was conducted (primary care, tertiary centre); (2) Size of study; (3) Duration of recruitment; (4) Duration of therapy; (5) Clinical endpoint: complete response versus partial response to therapy; (6) Short versus long-term response; (7) Follow-up after finishing therapy; (8) Quality of meta-analysis or systemic review (i.e. inclusion of abstract, contact of primary author, use of effective therapies to eradicate *H pylori*, etc); (9) Good clinical practice standards; (10) Publication bias (negative studies are less likely to be published); and (11) Publications before 1992 (definitions of dypspesia have changed, older studies were too small or used drugs that are not current any more). The issues to be addressed when interpreting trials of drug therapy in functional dyspepsia. In order to clarify issues, meta-analyses of various approaches to treat functional dyspepsia have been conducted. Interestingly, even meta-analyses performed in the same period have yielded completely opposite results[32,33]. Third, it is also very important to remember that functional dyspepsia tends to be a chronic condition[6,34]. In most studies therapy has been only given for short periods of time. There are essentially no studies evaluating chronic therapies for functional dyspepsia. And finally, the placebo effect in patients with functional dyspepsia is high. Therefore, most studies comparing a new therapy to no therapy will likely show a marked benefit for the treatment arm. But on the other side, clinicians treating patients with dyspepsia are many times confronted with the option of no therapy and any form of therapy, and will likely choose to give some form of therapy, because of the possibility of achieving a symptom response (similar or slightly better than placebo) in a significant percentage of patients.

Table 1 Drug therapies used in the therapy of functional dyspepsia

Acid suppression therapy
Proton pump inhibitors[1]
Omeprazole
Lansoprazole
H2 blockers
Cimetidine
Ranitidine
Famotidine
Simethicone
Herbal therapies
Iberogast (Iberis, peppermint, Camomille)
Artichoque leaf extract
Xinwei decoction
Ganaton (itorpide hydrochloride)
Capsaicin
Motility agents
Cisapride
Domperidone
Mosapride
Erythromicin
H pylori eradication
Antidepressants
Serotonin reuptake inhibitors (SSRI)
Amitryptiline
Placebo

[1]Other PPI such as esomeprazole, rabeprazole and pantoprzole are also used to treat functional dyspepsia, but the largest and most significant trials reported to date have used either omeprazole or lansoprazole.

CLINICAL PREDICTORS OF TREATMENT RESPONSE

In functional dyspepsia it has been suggested that there are associations between pathophysiologic mechanisms and symptoms (i.e. clinical presentation)[1-3,5]. These mechanisms include gastroesophageal reflux, delayed gastric emptying, impaired gastric accommodation to a meal, hypersensitivity to gastric distension, altered response to duodenal lipids or acid, abnormal duodenojejunal motility or central nervous dysfunction[1]. Therefore, it seems logical to hypothesize that the clinical presentation of patients with functional dyspepsia can guide therapy and even predict the response to therapy. The problem is that there is much overlap and interaction of these mechanisms, and their relevance for the individual patient is uncertain, especially because of the variability of symptoms over time[34]. Meineche-Schmidt *et al* showed that patients with functional dyspepsia usually have a combination of symptoms and the predominant symptom may vary over time[6]. Nonetheless, there are some studies which tried to address response to therapy based on the predominant symptom[35-37]. In a random

starting day trial including 301 patients with functional dyspepsia, Bytzer *et al* found that this sort of trial may be a valuable tool to identify response to acid suppression in dyspeptic patients[35]. In this study the only predictor of response was symptoms suggesting gastroesophageal reflux[35]. In another similar trial, Madsen *et al*[36] also found that response to therapy was more in patients with associated symptoms of gastro-oesophageal reflux[36]. Nonetheless, the same authors later showed that reproducibility of this study design was imperfect, mainly because of the strict criteria used to evaluate response and overall low response rates[37]. Lack of symptom stability in patients with functional dyspepsia further impairs the value of the random-starting-day trial and only patients with frequent and stable symptoms should be evaluated in this design[37].

H pylori ERADICATION

Although, the aim of our article is aimed mainly at describing drug therapies for functional dyspepsia, the role of *H pylori* therapy in patients with functional dyspepsia deserves a comment. Today it is clear that *H pylori* is the main cause of peptic ulcer disease but its role in non-ulcer dyspepsia is not well known. The effect of *H pylori* eradication on the symptoms of non-ulcer dyspepsia show somewhat conflicting results[2] Until the mid-nineties most studies evaluating *H pylori* eradication in the therapy of functional dyspepsia did not meet adequate methodological criteria. Since then there have been few well done prospective, randomized double-blind, placebo controlled trials of adequate size that evaluate the efficacy of *H pylori* eradication in functional dyspepsia[2,38-43] (Table 2). The ELAN study (European multi-centre trial) and a single-centre trial from Scotland suggested modest superiority of eradication therapy versus acid suppressive therapy with PPI alone[38,39]. The ELAN study further suggests that if patients were classified as responders (dyspepsia score of 1) or non-responders, there was a higher proportion of responders (44%) in patients with successful eradication compared to patients without eradication (36%)[38]. In the Scottish study it is likely that the high response occurred because of high prevalence of peptic ulcer disease[39]. The other studies evaluating elimination of *H pylori* in functional dyspepsia have had negative results[40-43] (Table 2). But recent well done meta-analysis and Cochrane Database systematic reviews show that there is a small but significant benefit of eliminating *H pylori* in patients with dyspepsia[44,45]. In the most comprehensive and thorough systematic review published to date, Moayyedi *et al* identified through electronic searches of the Cochrane Controlled Trials Register (CCTR), MEDLINE, EMBASE, CINAHL and SIGLE all randomized controlled trials evaluating *H pylori* eradication in functional dyspepsia and concluded that *H pylori* eradication therapy has a small but statistically significant effect in *H pylori* positive non-ulcer dyspepsia[44]. As demonstrated by Moayyedi *et al*[45], other systematic reviews have included trials that used drug regimens known to be ineffective for *H pylori* eradication, have failed to include recent trials, have not contacted authors for further information, have included abstracts, and have assumed that dropouts were treatment failures.

Table 2 Role of *H pylori* eradication in functional dyspepsia

Author	Name of study (if any)	Year	Type of study	No pts	Follow-up (mo)	Response
Mc Coll		1998	Singlecenter	218	12	Yes
Blum	OCAY	1998	Multicenter	438	12	No
Talley	ORCHID	1999	Multicenter	278	12	No
Talley		1999	Multicenter	170	12	No
Malfertheiner	ELAN	2001	Multicenter	180	12	Yes
Koelz	FROSCH	2003	Multicenter	181	6	No

ACID SUPPRESSIVE THERAPY

H2 receptor antagonists

Several studies and a large meta-analysis have demonstrated a slight advantage of H2-blockers over placebo or motility agents for treating functional dyspepsia[2,10-18]. In the large meta-analysis 22 studies met the inclusion criteria, 15 of which reported H2-blockers to be superior to placebo. Although many of the analyzed studies suffered from sub-optimal study design the odds ratio in favor of H2-blockers was 1.48 (95% confidence interval (CI): 0.9-2.3), for global assessment of dyspepsia symptoms, 2.3 (95% CI: 1.6-3.3) for improvement of epigastric pain, and 1.8 (95% CI: 1.2-2.8) for complete relief of epigastric pain. In most studies standard doses of H2-blockers were used but the duration of therapy was not longer than 8 wk[18]. Larger studies evaluating higher doses of H2-receptor antagonists and of longer duration may be necessary to determine the exact effect of H2-blockers on functional dyspepsia, but because PPI have been shown to be superior to placebo or H2-blockers in the therapy of functional dyspepsia, it is likely that we will not see major H2-blocker studies for the therapy of dyspepsia in the future[19-27].

Proton pump inhibitors

In a large primary care study, Meineche-Schmidt showed that in patients with presumed acid-related un-investigated dyspepsia, a standard dose of omeprazole 20 mg/d was significantly more effective than placebo in treating their self-worded main dyspepsia complaint[19]. In this study, 9% of the patients had sole reflux-like symptoms while the rest had other accompanying symptoms and would be considered to have dyspepsia. In fact, patients had on average more than five symptoms. As no initial endoscopic investigations were made, patients could have had a variety of possible diagnoses such as gastroesophageal reflux disease, ulcer disease, or functional dyspepsia. The recent CADET-PE study showed that if patients had endoscopic abnormalities (i.e. esophagitis), these were findings that predicted an appropriate response to acid suppression therapy[20].

The largest studies evaluating PPI for the therapy of functional dyspepsia are: the CADET-HN, OPERA,

Table 3 Role of acid suppression using PPI in functional dyspepsia

Author	Name of study	Year	Type of study	PPI	No pts	Follow-up	Response
Bolling-Sternevald	PILOT	1996	Multicenter	Omeprazole *vs* placebo	197	2 wk	Yes
Jones		1997	Multicenter	Lanzoprazole *vs* ranitidine	432	4 wk	L better than R
Talley	BOND	1998	Multicenter	Omeprazole *vs* placebo	642	4 wk	Yes
Talley	OPERA	1998	Multicenter	Omeprazole *vs* placebo	606	4 wk	No
Meineche-Schmidt	ENCORE	1999	Multicenter	Omeprazole *vs* placebo	567	3 mo	Yes
Peura		2004	Singlecenter	Lanzoprazole *vs* placebo	921	8 wk	Yes
Veldhuyzen	CADET-HN	2005	Multicenter	Omeprazole (O), ranitidine (R), cisapride(C), placebo	512	4 wk	O better than R, C or placebo

BOND, PILOT, ENCORE and lansoprazole studies[21-26] (Table 3). In the CADET-HN study, 512 *H pylori* negative patients were randomised to omeprazole, ranitidine or cisapride for 4 wk. The proportion of patients who were responders at 4 wk and 6-mo was significantly greater for those receiving omeprazole (31%), compared with cisapride (13%) or placebo (14%), and ranitidine (21%, $P=0.053$). In the BOND and OPERA multi-center trials 1262 patients with functional dyspepsia were randomized in a double-blind, placebo-controlled design in two studies with identical protocols to omeprazole 20 or 10 mg a day or identical placebo for 4 wk[21,22]. Complete symptom resolution was observed in 38% of patients taking omeprazole 20 mg/d, 36% on omeprazole 10 mg/d and 28% of patients on placebo. The Scandinavian Pilot study was a double blind, randomized, placebo controlled trial that evaluated the effect of gastric acid inhibition using omeprazole 20 mg twice daily in 197 patients recruited from gastroenterology practices in Bolling-Sternevald E *et al*[23]. Patients were excluded if they had a known gastrointestinal disorder, predominant symptoms indicating GERD, or the irritable bowel syndrome, as were patients with heartburn/regurgitation as their only symptom. Complete symptom relief was achieved in 32% of patients treated with omeprazole 20 mg/d compared with 14% of patients on placebo[23]. The ENCORE study addressed the consequences of relieving symptoms in patients with functional dyspepsia once they are off therapy[24]. In this study 567 patients from the OPERA study were followed for as additional three months during which time treatment was given at the discretion of the investigator. Responders had fewer clinic visits than non-responders (1.5 *vs* 2.0 mean visits) and fewer days on medication (mean, 9 d *vs* 23 d) over the 3-mo period (both, $P<0.001$). The quality of life in responders was better at study entry and persisted over 3 mo (all, $P<0.001$) Two large studies using lansoprazole have been published[25-26]. Jones *et al* performed a double-blind, parallel group, randomized, multi-center study in 32 general practices in the UK[25]. The investigators randomized 213 patients with symptoms of reflux-like or ulcer-like dyspepsia to receive lansoprazole 30 mg daily, and 219 to receive ranitidine 150 mg b.i.d. for 4 wk. After 2 wk 55% of the lansoprazole patients and 33% of the ranitidine group were symptom-free ($P=0.001$) with corresponding 4-wk figures of 69% and 44%, respectively ($P=0.001$). In the lansoprazole group, at 4 wk, 80% of patients were free of daytime heartburn and 81% of night-time epigastric pain, compared with 55% ($P=0.001$) and 65% ($P=0.01$) in the ranitidine group[25]. Peura *et al*[26] compared the efficacy of lansoprazole with placebo in relieving upper abdominal discomfort in 921 patients with functional dyspepsia in a blinded-randomized trial and moderate upper abdominal discomfort on at least 30% of screening days[26]. In contrast to the study of Jones *et al*, none of the patients had predominant symptoms suggestive of gastroesophageal reflux or endoscopic evidence of erosive or ulcerative esophagitis, or gastric or duodenal ulcer or erosion. Patients were assigned randomly to receive lansoprazole 15 mg ($n=305$), lansoprazole 30 mg ($n=308$), or placebo ($n=308$) daily for 8 wk. At wk 8, significantly greater mean reductions in the percentage of days with upper abdominal discomfort were reported in patients treated with lansoprazole 15 mg (35%) or 30 mg (34%) compared with those treated with placebo (19%) ($P<0.001$). Similarly, more patients treated with lansoprazole 15 mg (44%) or 30 mg (44%) reported complete symptom resolution at 8 wk than did placebo-treated patients (29%, $P<0.001$). Improvement of upper abdominal discomfort, however, was seen only in patients who had at least some symptoms of heartburn at enrollment[26].

Prokinetic agents

Although delayed gastric emptying is considered a major pathophysiological mechanism in functional dyspepsia, the efficacy of prokinetic drugs to treat this dyspepsia has not been clearly established[1-3]. Problems with studies evaluating prokinetic agents include small sample size, patient heterogeneity, and poor quality design. Nevertheless, prokinetic agents such as metoclopramide, cisapride, mosapride citrate, itopride hydrochloride and domperidone continue to be widely used for the therapy of functional dyspepsia worldwide[1,2,27-30,46-48]. Due to its severe cardiac side effects, cisapride is not currently available in most of Europe and North America. Recent reviews and meta-analyses suggest that domperidone and cisapride are more effective than placebo[49-51]. Veldhuyzen *et al*[49] performed a meta-analysis to determine the efficacy of cisapride and domperidone in functional dyspepsia in placebo-controlled trials that included more than twenty patients[49]. For cisapride, 17 studies met the inclusion criteria. For all outcome measures, there was a statistically significant benefit in favor of cisapride: global assessment of improvement by the investigator or patient, epigastric pain, early satiety,

abdominal distension, and nausea. There were insufficient data to determine whether there is a relationship between improvement in gastric emptying and response to treatment. For domperidone, four of eight studies could be used for the analysis of global assessment of improvement by the investigator. This showed an OR of 7.0 in favor of domperidone. Although both cisapride and domperidone appear to be efficacious in the treatment of functional dyspepsia, this conclusion was largely based on global assessment by the investigator, which is not an optimal outcome measure. The effect of other prokinetic drugs, such as erythromycin, alosetron and tegaserod on functional dyspepsia have been inconsistent[52,53].

Antidepressants

Antidepressants have been only marginally explored in functional dyspepsia. Serotonin re-uptake inhibitors (SSRI) have the potential of relieving functional dyspepsia because they increase the availability of synaptically released 5-HT (pro-motility) not only at the central nervous system, but also at the level of the enteric nervous system[1-3]. Amitryptilline and 5-HT-3 receptor antagonists have been investigated mainly in IBS and the few studies performed in functional dyspepsia have provided conflicting results[54-56]. Also, kappa-opioid receptor agonists might be useful for functional dyspepsia because of their antinociceptive effects, but available results in functional dyspepsia are inconclusive. Other receptors that represent potential clinical targets for antagonists include purinoceptors (i.e., P2X2/3 receptors), NMDA receptors (NR2B subtype), protease-activated receptor-2, the vanilloid receptor-1, tachykinin receptors (NK(1)/NK(2)) and cholecystokinin (CCK)(1) receptors[1,2].

Herbal Medications

Among the herbal medications, one of the best evaluated in prospective studies is Iberogast®[57,58]. Iberogast® is a combination of herbal medicines, which include Iberis, peppermint, and chamomile[58]. Meta-analysis of double-blind, randomized clinical studies that evaluated the efficacy of the herbal preparation Iberogast® in patients with functional dyspepsia demonstrated a clear and highly significant overall therapeutic effect of Iberogast® in the treatment of functional dyspepsia[57,59]. All studies had the same duration and used the same dosage of active treatment and the same primary outcome measure, a dyspepsia-specific gastrointestinal symptom score. Of the 592 trial participants, 196 were treated with Iberogast® and 192 with placebo or cisapride (positive control). The individual studies all showed a substantial improvement of symptoms with Iberogast® but varying results regarding its statistically significant superiority to placebo[57]. There are many other herbal medications used in the therapy of functional dyspepsia[59-61]. These include the traditional chinese herbal medicine Xinwei Decoction, ganaton (itorpide hydrochloride), capsaicin (red pepper) and artichoke leaf extract (ALE)[59-61]. It is paradoxixal to consider red pepper as a potential therapy for FD, but capsaicin, through its desensitation of gastric nociceptive C-fibers may alleviate dyspeptic symptoms[61]. Nonetheless, most studies using herbal medications are too small to allow us to reach conclusions regarding their efficacy to treat functional dyspepsia.

Hypnotherapy

Hypnotherapy was shown to be effective in irritable bowel syndrome, with long-term improvements in symptomatology and quality of life[62]. In another study aimed to evaluate the efficacy of hypnotherapy in functional dyspepsia, the data demonstrate an advantage of hypnotherapy over placebo and medical treatment[62]. Although hypnotherapy is time consuming and requires intense involvement by both physician and patient, it is an option that deserves further evaluation in future studies.

In summary, functional dyspepsia is a heterogeneous disease with variable response rates to therapy Although the main aim of therapy is to obtain complete and long-lasting relief of symptoms, in many patients there is only partial relief or the relief is only of short-term duration. Long-term improvement with drug therapy occurs in a minority of patients. Before starting drug therapy it is recommended to provide the patient with an explanation of the disease process and reassurance. Ruling out an organic cause of their disease by endoscopy can lead to improved acceptance of the disease process. In general, the approach to treat patients based on their main symptom is practical and effective. The general approach is to treat patients with acid suppressive therapy with proton pump inhibitors if the predominant symptom is epigastric pain or if there are gastroesophageal reflux symptoms present. The role of *H pylori* in functional dyspepsia continues to be a matter of debate. Nevertheless, recent data indicate that there is a modest but significant benefit in eradication of *H pylori* in functional dyspepsia. The overall data indicates that symptomatic improvement of patients with functional dyspepsia with current drug therapy is left with many unmet needs. Thus, more studies are needed to better understand this condition as well as to study more therapeutic possibilities.

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TOPIC HIGHLIGHT

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Functional dyspepsia: Are psychosocial factors of relevance?

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Abstract

The pathogenesis of Functional Dyspepsia (FD) remains unclear, appears diverse and is thus inadequately understood. Akin to other functional gastrointestinal disorders, research has demonstrated an association between this common diagnosis and psychosocial factors and psychiatric morbidity. Conceptualising the relevance of these factors within the syndrome of FD requires application of the biopsychosocial model of disease. Using this paradigm, dysregulation of the reciprocal communication between the brain and the gut is central to symptom generation, interpretation and exacerbation. Appreciation and understanding of the neurobiological correlates of various psychological states is also relevant. The view that psychosocial factors exert their influence in FD predominantly through motivation of health care seeking also persists. This appears too one-dimensional an assertion in light of the evidence available supporting a more intrinsic aetiological link. Evolving understanding of pathogenic mechanisms and the heterogeneous nature of the syndrome will facilitate effective management. Co-morbid psychiatric illness warrants treatment with conventional therapies. Acknowledging the relevance of psychosocial variables in FD, the degree of which is subject to variation, has implications for assessment and management. Available evidence suggests psychological therapies may benefit FD patients particularly those with chronic symptoms. The rationale for use of psychotropic medications in FD is apparent but the evidence base to support the use of antidepressant pharmacotherapy is to date limited.

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Key words: Functional dyspepsia; Psychosocial factors; Psychiatry; Pathophysiology

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INTRODUCTION

Dyspepsia, an umbrella term for a number of gastrointestinal symptoms, is common with prevalence rates estimated at 26%-41%[1]. The spectrum of dyspeptic symptoms may be defined as those arising from the upper digestive tract unrelated to colonic function[1]. Potentially caused by a variety of underlying conditions, most often, no overt cause is elucidated. In such cases, in the absence of demonstrable organic abnormality using available diagnostic measures, the term functional dyspepsia (FD) is employed. Non-ulcer dyspepsia (NUD) and essential dyspepsia are used synonymously. This syndromal diagnosis, according to the 'Rome II' Consensus Report, is defined by 12 wk in the past 12 mo, of persistent or recurrent pain or discomfort in the upper abdomen, in the absence of organic disease and no association with bowel habit. Using these guidelines, patients may also be categorised as ulcer-like dyspepsia, motility-like dyspepsia or unspecified dyspepsia[2]. Though highly prevalent, the underlying pathogenesis is incompletely elucidated, seems diverse and is thus, inadequately understood.

Some investigators object to the separation of functional gastrointestinal disorders (FGID's) into different clinical entities, and indeed, there is considerable overlap between FD and the oft more studied irritable bowel syndrome (IBS)[3]. In at least 40% of patients presenting to gastroenterologists this overlap in symptomatology is sizeable[4,5]. In fact, it has been suggested that the current evidence is insufficient to determine whether the two syndromes are indeed two distinct processes or simply different manifestations of a single, broad-spectrum condition[3]. Data in relation to the role of psychosocial factors in IBS is bountiful but as yet, there is less available pertaining to FD. Even so, as with IBS, psychosocial factors have long been implicated in the predisposition, precipitation and perpetuation of this syndrome.

Review of the literature clearly reveals an association between psychosocial factors and FGID's including FD[6-9]. However, the precise role of psychosocial variables in FD remains elusive. To explain this observation, one could espouse a number of hypotheses, none of which are likely to be mutually exclusive: Psychosocial variables are intrinsic to the aetiopathogenesis of FD; Psychosocial difficulties arise secondarily to chronic digestive symptoms; FD is a physical manifestation of unconscious psychological conflict or distress; Psychosocial factors motivate increased help-seeking and medical consultation; Psychosocial factors influence symptom experience.

Perhaps the most clinically useful way of conceptu-

alising a role for psychosocial factors in FD is within the biopsychosocial framework of disease as suggested by Drossman[10]. In this model, functional gastrointestinal disorders are seen as having mulitfactorial, bi-directional, rather than unicausal, unidirectional aetiology. This integrative view of illness, adopts the understanding that biological, psychological and social factors interact and said interaction accounts for the symptoms experienced, the patient's behavioural response and the outcome of the disorder. Many investigators now consider the 'Brain-Gut Axis' (BGA) to be the anatomical substrate, mediating this interaction[11]. At the very least, it provides a rough neurobiological framework for reciprocal connections between the brain and the gastrointestinal tract[12]. As a concept, it has emerged consequent to a convergence of research into brain-gut interactions and the effect of stress on such exchanges. The BGA is central to the biopsychosocial model in FD. To elaborate briefly, this axis is bi-directional and integrative with input from sensory sources (sight, smell, etc.) and somatosensory/viscerosensory sources, modified by cognitions and affects, and a neural circuit in the central nervous sysytem (CNS), the spinal cord, the autonomic nervous system (ANS) and enteric nervous system (ENS)[11]. Using this paradigm, chronic functional gastrointestinal symptoms can be seen as a result of dysregulation of intestinal motor, sensory and CNS activity, resulting from disturbance at some level of the BGA.

EVIDENCE TO SUPPORT AN ASSOCIATION BETWEEN PSYCHOSOCIAL FACTORS AND FUNCTIONAL DYSPEPSIA

A myriad of psychosocial factors have been examined in the literature in relation to FD. These include psychological distress, personality traits, social support, life-events and life-stresses including abuse and bereavement. The issue of Psychiatric Disorder co-morbidity is often addressed within the same context. The associations between a number of these variables and FD are outlined below.

Psychological morbidity

Studies have demonstrated a high prevalence of Psychiatric Disorder in patients with dyspepsia of unknown origin. In the midst of the debate concerning the significance of this relationship, speculation as to the potential for a shared pathophysiology has arisen[12]. The most common psychiatric co-morbidities in patients with functional dyspepsia are Anxiety Disorders, Depressive Disorders and Somatoform Disorders[6,13]. In a clinic based study, findings showed that 87% of patients with FD, compared to 25% of patients with organic dyspepsia, had a psychiatric diagnosis[13]. A larger study, utilising semi-structured psychiatric interview and psychometric tools, revealed that 34% percent of the FD patients versus 15% of the duodenal ulcer patients had a psychiatric diagnosis. Anxiety Disorders were again most common. A mere 1% of the healthy controls met criteria for a psychiatric diagnosis[6]. These findings are not consistent across studies but conflicting results may be explained by use of different sample populations. Interestingly, in contrast to the latter findings, an earlier study demonstrated no statistically significant difference between essential dyspepsia and duodenal ulcer patients on various psychometric test scores. However, elevated levels of neuroticism, anxiety and depression were demonstrated in FD patients compared to controls. No explanation was offered for lack of difference between the DU and FD patients[14]. Similarly, the results of a study examining psychosocial variables in organic dyspepsia and IBS, illustrated that the prevalence of formal psychiatric disorder did not differ significantly in both patient groups. The inclusion of chronic treatment resistant patients, who may account for the higher levels of psychopathology seen in other studies, was minimised by use of consecutive general practitioner referrals[15].

In a recent study, investigators sought to characterise relevant psychosocial variables and quality of life in a population of patients with functional dyspepsia, primarily referred from a primary care clinic, and in addition sought to correlate these variables with specific dyspeptic symptoms. It was hypothesised that if dyspeptic symptoms represented the physical manifestations of various aspects of psychological distress, a correlation should be evident between specific symptoms, measures of somatisation (the tendency to express emotional dysphoria as physical symptoms) and specific psychosocial variables. Self-report measures were used to evaluate symptomatology and psychological parameters. Results showed that, compared with healthy patients, patients with FD had significantly greater scores for dyspeptic symptoms, significantly greater psychiatric distress and significantly poorer quality of life. However, symptom severity and psychological distress did not correlate strongly. Thus, because of the lack of correlation, the authors concluded that digestive symptoms at that level of care are unlikely to represent physically manifested emotional distress. Instead, it would appear that dyspeptic symptoms are occurring against a background of psychiatric morbidity[16]. Studies, in addition to demonstrating greater psychiatric co-morbidity, have also revealed a greater tendency towards multiple extra-intestinal somatic complaints in FD patient groups[6]. This, together with overlapping symptoms in the various functional syndromes, contributes to the argument of proponents of a unitary somatisation syndrome[17].

Further studies have lent support to the notion that psychological distress, not necessarily warranting a psychiatric diagnosis, is involved in the causative pathway. Patients with NUD, at a clinic level, report more psychological distress than healthy controls[6,18]. However, a number of studies have demonstrated no psychological differences between people with functional bowel disorders who have not consulted a physician compared to community-based healthy controls[19]. This has engendered the assertion that psychosocial factors are not implicated in the aetiopathogenesis of FD and IBS, but rather serve to motivate health-care seeking i.e. act as confounding factors. More recently, in contrast to this dogma, elevated levels of psychological distress across all domains (except phobic anxiety) of the SCL-90-R, a measure of psychological state, have been demonstrated in a population-based study of subjects with functional GI disorders inclusive of both

consulters and non-consulters[9].

Life events

Life-events are changes in a person's life that require adjustment and where the demands associated with such events exceed resources available, stress is the result. The association between FD and stress in life has often been suggested, though yet again in a minority of studies, inconsistent results have been reported[20,21]. Severe life stress has been found immediately before the onset of functional bowel disorders[22] and may precede consultation with a general practitioner in dyspepsia sufferers[23]. It has been suggested however, that there is a lack of community studies that have carefully evaluated whether stress is linked to disease or merely to health-care seeking[9].

In a retrospective study using semi-structured interview and a number of psychometric tools, authors looked at the occurrence and perception of life events, personality factors and psychopathology of patients with FD, compared with DU patients and healthy controls. Results demonstrated that patients with FD experienced more life-events than both DU patients and controls. In addition, DU patients experienced more than controls. These findings were accounted for by differences in stressful life-events[7]. Moreover, the most prevalent types of severe events in all groups were changes in social activity, changes in job situation and illness in the family. Though, evaluation of stress is influenced by psychiatric co-morbidity, and indeed higher psychopathology scores were seen in the FD group, one could also argue conversely that the life-events precipitated the psychopathology. The authors suggest that the difference in perception of life-events may relate to an imbalance between stressors and coping abilities and that this may indirectly mediate gastric motor disturbances and abdominal discomfort. Similar findings were established in a clinic-based study which showed that highly threatening chronic difficulties were significantly more evident in the non-ulcerative dyspepsia group than controls, as were acute life-events which remained highly threatening one week after their onset[24].

A population based study, looked at a number of psychosocial variables, life-event stress inclusive, and their association with FGID's. Using the *life experiences survey*, it was demonstrated that subjects with higher negative change scores and greater total change scores were more likely to have a FGID. When the comparison was then limited to NUD patients only, subjects with higher positive change scores (indicating change with a positive impact) and higher total change scores were more likely to have NUD. Life-threatening events, in contrast to the latter study, were not associated with NUD[9].

Personality and coping style

In one study examining personality, investigators hypothesised that patients with FD would have more nervous symptoms and a different personality pattern than healthy control subjects[25]. Interesting findings were illustrated. Using a personality inventory, the patient group reported a lower *detachment* score than the control group, the *detachment scale* relating to the individual's need for social distance and coldness in social relations. This finding would indicate a greater need for attachment, which could conceivably lead to frustration. Female patients scored lower *socialisation* and higher *suspicion* than female controls. The authors suggest their findings may reflect a relationship between personality and development of gastrointestinal symptoms. Higher levels of introversion and suspicion may hinder adequate coping with stress.

Previous personality studies, showed significantly more symptoms of anxiety and tension and higher scores for trait tension and hostility for FD patients compared with peptic ulcer disease patients[18]. Others would suggest that personality differences are minimised when IBS co-morbidity is excluded. It has been shown that individuals with FD, without IBS, have higher scores of anxiety, neuroticism and depression, than paired community controls, but the differences are relatively small[14]. Where IBS is an accepted co-morbidity the differences are more significant[26]. Neuroticism, characterised by an individual' s tendency to have an exaggerated responsiveness to physiological changes, may theoretically exert an influence on the recognition and reporting of symptoms[27].

Coping response is another psychological factor that has proposed relations to FD symptoms. With regard to the relationship between coping behaviours and well being, FD patients may adopt a distinct coping pattern that is related to their heightened level of anxiety[28]. It has been shown that a non-discriminative, action orientated coping pattern was found to be characteristic of FD regardless of stressor controllability. Consistent use of this coping strategy under all circumstances may not be useful in mitigating distress, may provoke anxiety, may not be useful in altering outcome and may prove costly in terms of psychological strain.

Abuse

Studies suggest that abuse, both past and current, is an important risk factor for functional GI symptoms. It has been found that women outpatients with functional, as opposed to organic bowel disease, were nearly twice as likely to report sexual abuse[29]. Clinic-based studies may carry potential selection bias. However, a population based cross-sectional study, using a self-report questionnaire, has shown that subjects with an abuse history, in childhood or adulthood, were significantly more likely to report a history of dyspepsia or heartburn and that this association could not be explained by confounders for which the analysis accounted[30]. An interview-based study examining the prevalence of FGID's in women subject to ongoing abuse revealed that 71% had a FGID-67% having FD. This group also had higher levels of psychological distress[31].

These observations do not imply causality and the role of confounding co-morbidities to which the individual may be predisposed by virtue of the abuse, may be an issue[32]. It has also been observed that there is a high frequency of abuse history in other chronic pain conditions. Abuse may not be implicated in the aetiology of FGID but may be associated with a tendency to communicate psychological distress through physical symptoms[33].

Psychosocial factors and health-care seeking

Aetiological issues aside, it is reasonable to hypothesise

that psychosocial factors may also exert their effect in FD through an influence on health-care seeking. Health-care seeking is determined by a plethora of factors including symptom severity, personality, coping skills, social support, sociodemographics, psychological morbidity and health-care availability[34]. Illness behaviour, which may be defined as how one perceives, evaluates and acts upon symptoms[35], is also critically involved in the decision to seek medical consultation. Intrinsic to this process are illness beliefs and attitudes. It has been suggested that the observed association between FD and psychosocial factors is in fact mediated through increased help-seeking prompted by elevated levels of psychosocial distress. This theory is derived from the findings by some investigators, in volunteer studies, identifying no differences across psychological parameters between healthy controls and FD patients who have not sought medical advice[36,37].

A number of studies have demonstrated elevated levels of psychosocial distress amongst consulters recruited at clinic, in comparison with non-consulters, with functional dyspeptic symptoms. One such study showed that the former have more worries about cancer and heart disease than the latter. Consulters interviewed also had experienced more stressful or threatening life-events over the previous 6 mo[38]. These are factors that may potentially motivate health-care seeking to ameliorate concern and gain reassurance. More recently, a critical review of the literature pertaining to predictors of health-care seeking for both IBS and FD, was undertaken. Authors concluded that psychosocial factors including life-event stress, psychological morbidity, personality, abuse, and abnormal illness attitudes and beliefs have been found to characterise those that seek help versus those that do not[34]. In a study that sought to examine the differences in behavioural and perceptual characteristics between non-consulters and consulters with FD in a Chinese population, it was found that non-consulters were distinguishable by their perceptual style, coping behaviours and psychological symptoms. Moreover, investigators found levels of anxiety and depression to be highest in consulters compared to non-consulters and healthy controls[39].

Others refute the generalisability of these findings. The results of a recent community based study, suggest that psychosocial factors are significantly associated with functional GI disorders in both consulters and non-consulters, at population level[9]. The debate thus clearly remains unresolved.

ABNORMALITIES OF FUNCTION IN FUNCTIONAL DYSPEPSIA

Though the pathophysiology underlying FD is incompletely elucidated and seems heterogeneous, several mechanisms to explain symptom manifestation have been proposed. Motor abnormalities of the upper gut have been demonstrated including delayed gastric emptying, antroduodenal dysmotility and altered gastric compliance[40,41]. So too has gastric sensory dysfunction in the form of visceral hypersensitivity[42,43]. Infective and inflammatory processes have also been proposed which may involve *Helicobacter pylori* and acute gastric infection[44-46]. Different types of inflammation throughout the gut can leave a legacy resulting in abnormal function in apparently histologically normal organs, by means of proposed mechanisms including: defective resolution of inflammation, changes in mucosal function and persistent changes within the ENS[44]. In a review of the molecular basis of FGIDs, it has been suggested that mediators or regulators of mucosal inflammation, including cytokines, may trigger events that ultimately result in the manifestation of functional gastrointestinal disorders[47]. Abnormalities of receptor function, including central serotonin receptors have also been implicated[48,49].

Influence of psychosocial factors on abnormalities of function

Using the brain-gut interaction construct, how then do psychosocial factors ultimately influence FD symptomatology? Stress is a characteristic shared by the various psychosocial factors that have a demonstrated association with FD. From a psychological perspective, stress is emergent when demand exceeds resources. More broadly, it is defined as 'any threat to the homeostasis of an organism, be it real or perceived, which may be posed by events in the outside world or within[50]. In recent times, the biological substrates of the stress response, in health and disease, have been recognised. In brief summary, centrally, interoceptive (systemic) stressors employ subcortical circuits, while exteroceptive (psychological) stressors engage pathways in the limbic forebrain, hippocampus and amygdala. Both circuits activate hypothalamic effector neurons. Corticotrophin releasing hormone (CRH), not only secreted in the hypothalamus, is an important mediator of the central stress response. The latter is generated by a network of integrative brain structures, in particular subregions of the hypothalamus, amygdala and periaqueductal grey. Mayer refers to this central circuitry as the 'emotional motor system' (EMS), which is under feedback control via ascending monoaminergic projections and circulating glucocorticoids. Outputs from the EMS play a role in mediating the peripheral response[50]. Neuro-endocrine systems are heavily implicated and include the hypothalamic-pituitary-adrenal (HPA) axis[51]. The autonomic nervous system and endogenous pain modulation system are also involved[50]. It is generally recognised that early life-trauma and chronic severe stress in adulthood can cause long-lasting, potentially irreversible, changes in the stress response system[52]. It is possible therefore that in vulnerable individuals the neurobiological substrates of stress, or maladaptive states thereof, operating on the BGA, form part of the link between psychosocial factors and FD.

Approximately one in three FD patients have a definable gastric motor abnormality[53]. In patients with FD, the effect of stress on antro-duodenal activity has been studied, revealing findings contrary to those seen in healthy controls[54]. In an experimental design, it was shown that antro-duodenal motility was diminished by stress in healthy controls. This was not the case in dyspeptic patients suggesting that FD may arise from the effect of stress on upper gut motility in susceptible individuals. The autonomic nervous system has been proposed as the

mediating mechanism for this effect. In an earlier study, antral motility and autonomic function in FD patients and healthy controls were investigated[55]. The effect of mental stress on antral motility was also examined. Findings confirmed autonomic dysfunction, specifically low vagal tone, in functional GI disorders. At baseline, post-prandial antral motility was on average reduced in FD patients compared to controls. Moreover, acute mental stress diminished post-prandial antral motility in healthy subjects but not in dyspeptics. In fact, some patients demonstrated increased motility. Subsequently, it was proposed that poor vagal tone may represent a mediating mechanism for the causal effects of personality on antral motility and related FD symptoms[56]. The relationship among a number of psychological factors, antral dysmotility and dyspeptic symptoms in FD patients were examined. Specifically, it was asked if any such relationship depends on vagal activity, by examining the relationship between these psychological factors and vagal tone. Findings showed that psychological predictors explained a substantial amount of the variance in task-related dyspeptic symptoms, vagal tone and gastric antral dysmotility. FD patients had lower scores on vagal tone and motility index in addition to the expected higher scores on epigastric discomfort. Symptoms appeared to be predicted by trait anxiety, depression and neuroticism and poor vagal tone was associated with neuroticism.

Several studies have established that patients with FD have enhanced sensitivity to gastric distension thus potentially allowing physiological stimuli to induce symptoms[43,57]. The mechanisms underlying visceral hypersensitivity are not fully clarified but there are several sources of evidence for a role of the central nervous system. Studies in experimental animals indicate that acute psychological stress seems to facilitate increased sensitivity to visceral stimuli[40]. Conceivably, psychosocial stressors may affect visceral sensation through an effect on central processing and/or modulation of afferent visceral information or on central cortisol receptors. With reference to the latter, a thorough characterisation of the HPA axis in FGID has not been reported. However, it has been demonstrated that chronic stress may result in HPA axis overactivity and thus hypercortisolaemia[52]. Brain regions involved in visceral sensation, and indeed mood regulation, express cortisol receptors and abnormal levels of cortisol can causes changes in these structures ultimately resulting in abnormal visceral sensation, affective disorders or both[12,50]. Imaging research has revealed considerable overlap between brain regions involved in processing visceral sensation and those important for emotional regulation[12,58,59]. From animal research, neonatal maternal separation in rats, a model of early-life stess, predisposes them to develop visceral hyperalgesia in response to psychosocial stress[60]. It is felt that this is probably CRH mediated.

Approximately 25% of FD cases develop acutely after an acute infectious illness often characterised by vomiting and fever[46]. It is well recognised that stress plays a role in immunological modulation[61]. Chronic stress renders individuals more vulnerable to intestinal infection and inflammation, which can be an important pathophysiological mechanism in post-infectious FGID[62].

PSYCHOSOCIAL FACTORS AND MANAGEMENT OF FUNCTIONAL DYSPEPSIA

The association between psychosocial stressors and FD and the high rates of psychiatric co-morbidity in FD, clearly have implications for effective management. Taking a psychosocial history may help reduce return visits in IBS patients and this may also apply to other FGIDs[63]. Depression and anxiety have been shown to adversely affect outcome in IBS[64] as have pronounced health worries and poor psychosocial support. Overt psychiatric illness should respond to conventional treatment and if suspected, or if there is evidence of somatisation in other systems leading to multiple consultations across specialities, referral to a mental health profession may be considered[8]. As with IBS, treatment in FD is symptom driven. In reducing the likelihood of invalidating the patient's symptoms (secondary to lack of organic pathology) it may help to explain the mechanisms underlying brain-gut interactions. Acknowledging the relevance of psychosocial factors will no doubt meet with some resistance in certain patients.

If the role of psychosocial factors in FD remains to be fully clarified, then the role of psychological therapies is similarly undetermined. However, patients with FD may benefit from psychological treatments. This is particularly true of those with chronic symptoms unresponsive to conventional medical approaches. Psychotherapy has been defined as an interpersonal process designed to bring about modifications of feelings, cognitions, attitudes and behaviour, which have proved troublesome to the patient seeking help from a trained professional[65]. There are wide ranges of interventions that can be described as psychotherapeutic including cognitive-behavioural therapy (CBT), psychodynamic psychotherapy and group therapies. Psychodynamic therapies focus on how maladaptive ideas and behaviours have emerged whereas cognitive behavioural work concentrates on how maladaptive ideas and belief systems are maintained by the patient's environment and behaviour[66].

A systematic review to establish the effectiveness of psychotherapy and hypnosis in FD, revealed a paucity of randomised controlled trials investigating psychological intervention in FD[66]. The data available suggest that these techniques benefit FD but the authors conclude that the evidence is insufficient to confirm their efficacy. One particular study randomised patients to psychodynamic-interpersonal psychotherapy or supportive therapy, which consisted of sympathy and support for the same duration of time as the intervention arm. Patients recruited had chronic symptoms and a history of failed response to conventional pharmacological therapy. After 12 wk of treatment the intervention group were significantly better across parameters but after 12 mo, the symptom scores were similar between groups[67]. CBT has been found to be effective in the treatment of depression, anxiety disorders and of patients with medically unexplained symptoms[68]. A study comparing the outcome of cognitive therapy (attempts to change dysfunctional thoughts and behaviour

by cognitive restructuring, behavioural modelling and role-play) and a control group intervention (bimonthly therapist visits), in patients with FD, demonstrated significantly greater improvement in the former[69].

The rationale for using psychotropic medication rests in the level of psychiatric co-morbidity seen in patients with FD as mentioned earlier. Additionally, there is data to support the use of antidepressants in the relief of chronic pain[8]. A meta-analysis of the treatment of functional gastrointestinal disorders with antidepressant medications, identifying 3 randomised controlled trials evaluating FD, suggested that antidepressants (predominantly tricyclics) might reduce the symptoms of FGIDs. Of note, the majority of studies included, used antidepressant doses that are essentially sub-therapeutic for treatment of depression. The authors thus suggest that it is unlikely that the benefit observed is due entirely to the antidepressant effect of these drugs[70]. There is little data available on the use of selective serotonin reuptake inhibitors. It seems reasonable that choice of drug is tailored to the individuals dominant symptom pattern whilst remaining cognisant of side-effect profiles. Essentially, if used, psychotropics should be regarded as complimentary to an overall multi-component plan.

In conclusion, functional dyspepsia is a heterogeneous disorder in which the role of psychosocial factors continues to be the subject of debate. The suggestion that psychological morbidity and social stressors serve to motivate physician consultation, rather than possessing an aetiological role in symptom pathogenesis, has been challenged. Despite inconsistent findings, it is evident that there is a high level of psychiatric co-morbidity with FD and that anxiety disorders predominate. Individuals with FD appear to experience a greater number of life-events, which are not limited to those of negative impact, compared to healthy controls. A recent or remote history of abuse is a non-specific risk-factor for symptoms of FD. The speculation that functional gastrointestinal disorders and psychopathology share common pathophysiology is interesting and warrants further examination. The evolving appreciation and delineation of brain-gut interactions and CNS processing continues to enhance our understanding of the complex mechanisms underlying symptom generation, interpretation and presentation.

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TOPIC HIGHLIGHT

Andrew Seng Boon Chua, MD, *Series Editor*

Functional dyspepsia and irritable bowel syndrome, are they different entities and does it matter?

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Abstract

A high prevalence of overlap between functional dyspepsia and irritable bowel syndrome has been consistently and universally reported. Recent studies demonstrating shared common pathophysiological disturbances including delayed gastric emptying and visceral hypersensitivity involving more than one region, suggest that these patients have a generalised rather than regional, disorder of the gut. Furthermore, a study of the natural history of dyspepsia suggests that with time, a substantial proportion will evolve into IBS. The recognition of IBS in dyspeptic patients has potentially profound therapeutic importance. It could help to reduce the risk of unnecessary cholecystectomy in IBS patients. The ability to appreciate the extent of involvement could allow us to address the disturbances more comprehensively, and thereby achieve greater patient satisfaction with their treatment.

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Key words: Functional dyspepsia; Irritable bowel syndrome; Common pathophysiology; Asian; Epidemiology

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INTRODUCTION

Many patients with functional dyspepsia (FD) will also fulfil the criteria for irritable bowel syndrome (IBS). Functional dyspepsia (FD) may coexist with irritable bowel syndrome (IBS) for several reasons. The association may be a chance occurrence simply because both FD and IBS are so common. IBS patients with upper abdominal pain may be mistakenly labelled as FD. Both conditions could share common pathophysiological disturbances.

PREVALENCE OF FD-IBS OVERLAP

In population-based studies, the estimated prevalence of IBS among dyspeptic subjects, ranges between 13% and 29%, while the prevalence of FD among IBS subjects ranges between 29% and 87%[1-3]. These figures are higher than the corresponding figures in the general population, where the prevalence of IBS and FD are estimated to be about 10% and 20% respectively. This would suggest that the overlap is not simply the chance occurrence of two extremely common human conditions. In patient-based series, as opposed to community series, the prevalence of overlap appears to be even higher, with between 26% and 46% of FD patients having concomitant IBS and as many as 87% of IBS patients having concomitant FD[4-8]. This observation would appear to suggest that the coexistence of these two sets of symptoms increases the likelihood of a subject seeking medical attention. There is some support for this from a study by van Bommel *et al* who found in a survey of primary care patients consulting for dyspeptic complaints, that those who had concomitant IBS were most likely to be referred for specialist assessment[9].

In Asia the figures are similar. In a population-based study from Mumbai, India, the prevalence of dyspepsia was 30%, while among subjects with IBS, the prevalence of dyspepsia was 58%[10]. Similarly, the prevalence of IBS among subjects with dyspepsia at 14% was greater than in the general population where it was 7.5%. In a community study from Xi'an in northwest China, Wang *et al*[11]found that significantly more subjects with GERD symptoms also experienced constipation (22%), and diarrhoea (14%) than subjects without GERD symptoms (10%, 5% respectively). In the Guangdong province of south China, Chen *et al* reported that 22% of 473 FD patients attending gastroenterologists had concomitant IBS, whereas the prevalence of IBS in this population was estimated to be 7%[12]. In the Zhejiang province of China, the prevalence of FD among 662 consecutive IBS patients consulting gastroenterologists, was 64%[13].

IBS MISDIAGNOSED AS FD

It is conceivable that IBS patients who present with upper abdominal pain may be mislabelled as FD. Colonic distension studies have clearly demonstrated that in IBS patients, pain from the colon can be referred to the upper abdomen. In a classical experiment, Swarbrick *et al* provoked abdominal pain in 48 IBS patients by using an attached balloon to distend the colon at various regions during colonoscopy[14]. Distension of the mid transverse colon induced pain in the epigastrium in 10 patients.

There are several reasons why doctors may mislabel IBS patients as having dyspepsia and overlook the association with bowel disturbances. Past diagnostic criteria for IBS have tended to focus on the bowel disturbances and lower abdominal pain. Even the Rome II criteria did not consider the association of pain with a meal as a possible symptom of IBS. And yet, a study by Ragnarsson *et al* suggested that about 50% of IBS patients would experience postprandial pain[15]. Foods that are commonly implicated in dyspepsia include spicy foods such as chilli, fatty foods and coffee. In a study of chronic upper abdominal pain, Kang *et al* found that as many IBS patients as FD patients reported precipitation of pain by fatty foods (28%, 19%), chilli (45%, 47%) and coffee (41%, 36%)[16]. A study by Simren *et al* has demonstrated that lipid infusion into the duodenum increased the area of referred pain in IBS patients such that more patients were experiencing pain in the upper abdomen during distension of the sigmoid colon[17].

One important reason for differentiating IBS from dyspeptic symptoms, is to avoid the risks of unnecessary surgery. Several recent studies have highlighted the risks of unnecessary surgery in IBS[18-20]. Based on statistics from studies in the USA population an estimated 8% of IBS patients will undergo unnecessary cholecystectomy in a lifetime[20]. It is possible that in some of these IBS patients undergoing cholecystectomy, their IBS symptoms could have been mislabelled as dyspeptic symptoms. In a study of 22 patients with chronic right upper quadrant pain, their pain was reproduced by distension of the jejunum in 15, ileum in 12 and right colon in 9, and 16 had symptoms consistent with IBS[21]. And yet, prior to their referral these patients had never received a diagnosis of IBS. On the contrary, they had been subjected to an average of 3 pancreatobiliary investigations each, 10 of them had been subjected to cholecystectomy without obvious improvement, even in the 5 who proved to have gallstones. The risk of cholecystectomy could be particularly greater among subjects with FD-IBS. In a community survey Talley *et al* found a cholecystectomy rate of 19% in FD-IBS subjects compared with only 6%-8% for subjects with either FD alone or IBS alone, no different from subjects with neither IBS nor FD[1].

The mislabelling of IBS as dyspepsia is likely to be a greater problem in Asia than in the west. IBS patients in Asia appear to present more commonly with upper abdominal pain[22]. In numerous studies from India, more than half of their patients complained of upper abdominal pain, whereas in western series only about a quarter do so. In a recent study from Taiwan, Lu *et al*[23] looked at 481 patients with FD by Rome criteria, and found more than half had IBS criteria. When they looked at these patients with FD criteria and IBS criteria, they found that in about a third of these patients, their upper abdominal symptoms could be attributed solely to IBS alone. In a study from the same centre in Taiwan, IBS subjects were observed to have twice the rate of cholecystectomy of the non-IBS subjects[24].

In the east, another possible reason for misdiagnosing IBS as dyspepsia could be the milder degree of defecatory and stool disturbances. In a community study from Singapore, more than half of IBS subjects reported pain in the upper abdomen[25]. Despite the fact that 50% fulfilled criteria for chronic constipation and 25% for chronic diarrhoea, when these people were asked to describe their bowel pattern, 77% thought that they had a normal bowel habit.

SHARED COMMON PATHOPHYSIOLGICAL DISTURBANCES

Pathophysiological processes implicated in FD such as altered gastric emptying and visceral hypersensitivity, have also been demonstrated in IBS patients. Post-infectious IBS is a well recognised entity. A recent study suggests that FD could also arise post-infection.

Delayed gastric emptying

Although early studies failed to demonstrate delayed gastric empyting among IBS patients, recent studies employing greater number of patients suggests that it exists in about 30% of IBS patients, particularly in those with constipation-predominant IBS[26-32]. Van Wijk *et al*[29] studied 16 patients with constipation predominant IBS and found slower gastric emptying of solids compared with healthy controls. Evans *et al*[30] studied 44 IBS patients and observed delayed gastric emptying in 9 of 22 (41%) C-IBS and 8 of 22 (36%) D-IBS patients. Caballero-Plasencia *et al*[31] studied 50 IBS (30 C-IBS, 20 D-IBS) patients and found evidence of delayed gastric emptying of both solids and liquids compared with healthy controls. IBS patients with constipation had slower gastric emptying of solids compared with diarrhoea-predominant IBS.

Recently Stanghellini *et al* studied gastric emptying in a large cohort of IBS patients[32]. In 146 IBS patients, majority IBS with constipation, overlapping FD was present in 96 patients (66%). Gastric emptying was delayed in patients with concomitant FD but not in those with IBS alone. In particular, postprandial fullness and nausea were independently associated with delayed gastric emptying. In another large study involving 309 FD patients, Corsetti *et al* using the gastric emptying breath test, found evidence of delayed gastric emptying to solid meal in 23% of patients with FD-IBS and 19% of patients FD alone[8].

Visceral hypersensitivity

In the study by Corsetti *et al*[8] using the gastric barostat, impaired accommodation to a meal was found to be as prevalent in FD-IBS patients (31%) as in FD patients (35%). However, significantly more FD-IBS patients were found to have hypersensitivity to gastric distension than patients

with FD alone (44% *vs* 28%). On the other hand, testing of visceral sensitivity in other regions of the GI tract has not shown any significant differences between FD and IBS subjects. Holtmann *et al* tested sensitivity to distension in the third part of the duodenum and demonstrated hypersensitivity in patients with FD alone, IBS alone and FD-IBS, with no significant differences observed between the groups[33]. Trimble *et al*[34]demonstrated hypersensitivity to distension of the rectum and the oesophagus in both IBS and FD patients compared to healthy controls. However, patients had normal pain thresholds to electrocutaneous stimulus applied to the finger.

Post-infection sensitization

The development of IBS following an episode of acute gastroenteritis has been documented in prospective studies[35-36]. Retrospective studies had previously suggested that FD could also develop post-infection, and that this form of FD was associated with delayed gastric emptying and impaired accommodation[37]. While post-infectious IBS has been largely associated with bacterial infections such as salmonella and campylobacter, post-infectious dyspepsia was presumed to be of viral origin. Recently however, a large prospective study of a well documented single source out-break of salmonella gastroenteritis demonstrated the development not only of IBS, but also for the first time, of dyspepsia[38]. The three most common dyspeptic symptoms were pain, bloating and fullness. Prolonged abdominal pain and vomiting during the acute episode were found to be positive predictors. In patients who developed IBS, there was a 62% overlap with FD at 12 mo post-infection. In patients who developed FD, there was a 46% overlap with IBS.

SYMPTOMATOLOGY OF FD-IBS

The type of symptoms that appear to predominate among FD-IBS subjects, as well as the substantial flux between FD and IBS populations observed in longitudinal studies, lends further support to the hypothesis for a shared common pathophysiology between FD and IBS.

In a Swedish community-based study Agreus *et al* found that the greatest overlap was between IBS and dysmotility-like dyspepsia[3]. In Italy Stanghellini *et al* studied a series of 483 patients with FD, and found that patients with predominantly non-painful symptoms such as post-prandial fullness, nausea and vomiting, were more likely than patients with predominantly epigastric pain to be associated with IBS[6]. Similarly in Singapore, Gwee *et al*[39]studied a consecutive series of 224 patients with FD, and found that 33% had concomitant IBS, with dysmotility type symptoms predominating in the FD-IBS group.

Patients with constipation predominant IBS (IBS-C) appear to be more prone to FD than diarrhoea predominant IBS (IBS-D), and their dyspeptic symptoms appear to be of the dysmotility type. Schmulson *et al*[40] found that IBS-C patients had more upper GI symptoms, in particular early satiety and postprandial fullness, than IBS-D. Similarly, Talley *et al*[7] reported that more patients with IBS-C had upper abdominal pain, and in particular, had significantly more bloating and early satiety than IBS-D. In China, there was a trend for a greater prevalence of FD among IBS-C patients (70%) than IBS-D patients (62%)[13]. A cologastric brake has been proposed by Tjeerdsma *et al* as one mechanism through which constipation could give rise to upper abdominal symptoms[41]. Delayed gastric emptying was recorded in a study of healthy volunteers who had suppressed defecation for 3 d.

The available literature suggests that FD-IBS patients could have more severe symptoms, and it is possible that IBS could be a predictor of consultation seeking behaviour. Talley *et al* reported that community subjects with FD-IBS subjects had made more physician visits for abdominal pain and for disturbed defecation than subjects who had either FD or IBS alone[1]. Furthermore, while the rates of appendicectomy and cholecystectomy among subjects with FD alone or IBS alone were no different from subjects with neither FD nor IBS, the rates were two to three times greater for subjects with FD-IBS. In a study of patients consulting their general practitioners for dyspepsia, it was also found that those who had IBS along with their dyspeptic complaints were most at risk of being referred for specialist assessment[9]. Corsetti *et al*[8] reported that FD-IBS was associated with higher symptom severity scores.

Evolution of Symptoms

In a one year study of symptom turnover by Agreus *et al* 87% of subjects with IBS also fulfilled Rome I criteria for dyspepsia[13]. When these IBS subjects were surveyed one year later, 50% still fulfilled IBS criteria. However, 22% appeared to lose their IBS, but changed their symptom profile to one of dyspepsia. Among subjects who were categorised as dyspepsia, 43% retained this diagnosis, while 16% converted to IBS. By comparison, among subjects who were symptomless at the beginning of the survey, 80% remained asymptomatic, while only 1% developed IBS and 3% developed dyspepsia. When this study was extended to 7 years Agreus *et al*[42] observed that dyspepsia appeared to decrease with advancing age, the prevalence in their cohort declined from an initial 11.7% to 8.1%, whereas IBS increased in prevalence from 8.9% to 13.6%. Of all subjects with dyspepsia on the first survey, only 30% could still be classified as dyspepsia after 7 years, 17% became asymptomatic, while 18% evolved to IBS. With IBS, 55% remained as IBS, 13% became asymptomatic, and only 8% evolved to dyspepsia. Whereas substantial symptom fluctuation and symptom profile flux was observed between IBS and dyspepsia, the prevalence of GERD remained relatively stable, and only a minority (<10%) of GERD evolved to dyspepsia or IBS, or vice-versa. Thus, while patients with GERD appear to form a distinct population from FD or IBS, separation between the latter two blurred with time.

In conclusion, in view of the high degree of symptom overlap it has been suggested that the separation of functional GI disorders into FD and IBS may be inappropriate[3]. Given the shared pathophysiology, some have suggested that patients with FD-IBS have an irritable gut[3,34]. With the substantial flux between FD and IBS, and the observation that a greater proportion of dyspeptic patients

evolved into IBS than the reverse, it is tantalizing to speculate that FD could be a precursor of IBS, and eventually all FD could evolve into IBS. At the end of the day, the key consideration is whether the division of patients into FD and IBS has led to improved treatment outcomes. The experience with sub-classification of FD suggests that this is not the case. Perhaps it is now time to lump again rather than split, and also in our research to return to the old approach of examining combination treatments. After all, combination treatment is the reality of clinical practice.

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REVIEW

Latest insights into the effects of *Helicobacter pylori* infection on gastric carcinogenesis

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Abstract

There appears to be the strong association between *Helicobacter pylori* (*H pylori*) and gastric cancer. We reviewed the latest evidences about the effects of *H pylori* infection on gastric carcinogenesis, classified into epidemiology, dynamics of gastric mucosal changes, DNA damages, virulence factors, host factors, and source of gastric malignancy. Through the considerable progress made in research into virulence factors resulting from differences between *H pylori* strains, such as *cagA* positivity, as well as into host factors, such as gene polymorphisms, a diverse spectrum of *H pylori*-associated diseases, including gastric cancer, is beginning to lend itself to elucidation. The impact of the novel hypothesis advanced by Houghton *et al* proposing bone-marrow derived stem cells (BMDC) as a potential source of gastric malignancy on evolving research remains to be seen with interest. Further progress in research into *H pylori* eradication as a viable prophylaxis of gastric cancer, as well as into the mechanisms of gastric carcinogenesis, is to be eagerly awaited for the current year and beyond.

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Key words: *Helicobacter pylori*; Gastric cancer; Carcinogenesis; *CagA*; Intestinal metaplasia

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INTRODUCTION

By now, there appears to be no doubt about the strong association between *Helicobacter pylori* (*H pylori*) and gastric cancer, and the focus of current research interest has now shifted to the mechanism of gastric carcinogenesis associated with *H pylori* infection, long regarded as a black box, which is now beginning to be unraveled thanks to the considerable progress made in this area through the clarification of various aspects including *cagA*. Difficult as it is to cover the full spectrum of papers accumulated on *H pylori* and gastric cancer even in the last few years, in what follows, we propose to provide a review of the literature, while drawing on selected papers of interest on this topic.

EPIDEMIOLOGY

In the beginning of 2004, Wong *et al*[1] reported on the results of a randomized controlled trial investigating the association between *H pylori* eradication and prophylaxis of gastric cancer in 1630 patients with *H pylori* infection (of whom 988 patients were found to have no precancerous lesions, such as atrophy of gastric mucosa, intestinal hyperplasia, or atypical epithelium) allocated to either the eradication arm or placebo arm and followed up for 7.5 years. Of the patients who developed gastric cancer during follow-up, 7 had had *H pylori* eradicated, and 11 had received placebo, and there was no significant ($P=0.33$) difference in the incidence of gastric cancer between the two arms. However, a subset analysis of patients without precancerous lesions in both the treatment and placebo arms revealed that the incidence of gastric cancer was 6 in the placebo arm *versus* 0 in the treatment arm ($P=0.02$), suggesting that *H pylori* eradication significantly inhibited the incidence of gastric carcinogenesis in patients without precancerous lesions.

In another randomized, double-blind, placebo-controlled trial in 248 patients, of whom 122 received eradication, and 126 received placebo, Ley *et al*[2] reported in their follow-up ranging from 6 wk to 1 year that there was no significant improvement with *H pylori* eradication in the consensus "worst biopsy" diagnosis, while there was improvement in the weighted index score, and concluded that the study results do not prove that eradication of *H pylori* decreases cancer risk. However, these inconclusive

results may be attributable to the short-term follow-up of the study.

The Japan Collaborative Cohort (JACC) Study Group for Evaluation of Cancer Risk reported that women with a family history of gastric cancer and infected with *H pylori* were associated with a 5.10-fold risk for gastric cancer compared to women without a family history of gastric cancer and *H pylori* infection[3]. In contrast, family history and *H pylori* infection were not found to be significant risk factors for gastric cancer in male patients in their report, where the investigations of *H pylori* infection were performed using only *H pylori* antibodies.

Matsuhisa *et al*[4] investigated the ratio of corpus gastritis to antrum gastritis (C/A ratio) in *H pylori*-positive Chinese, Thai and Vietnamese adult populations compared to the Japanese population by evaluating the degree of neutrophil activity in the corpus and antrum of these patients. The C/A ratio was found to be significantly higher in the Japanese population compared to the other populations, where corpus-predominant gastritis was characteristic of aged Japanese and Chinese (Fuzhou), while, in contrast, gastritis in Chinese (Beijing), Thai and Vietnamese was found to be antrum-predominant, which is considered to be related to the low incidence of gastric cancer in Thai and Vietnamese.

Brenner *et al*[5] demonstrated in a case-control study that after excluding those who met the exclusion criteria (blood sample taken more than 90 d after gastrectomy, advanced (T4) gastric cancer, and *cagA* positivity in Western blot analysis despite a negative result in anti-*H pylori* immunoglobulin G enzyme-linked immunosorbent assay), as well as those who had borderline levels in immunoglobulin G enzyme-linked immunosorbent assay, the odds ratio for noncardia gastric cancer was increased from 3.7 to 18.3 in those with any *H pylori* infection, and from 5.7 to 28.4 in those with *cagA*-positive *H pylori* infection. The authors therefore concluded that *H pylori* infection might even be a (close to) necessary condition for the development of nocardia gastric cancer.

In a multi-center trial evaluating 2 503 patients with histologically confirmed gastric cancer *versus* 6 578 control subjects, Kato *et al*[6] demonstrated that *H pylori* infection was strongly associated with the development of gastric cancer ($OR = 2.47$), while there were no significant differences in the prevalence of *H pylori* infection, histological subtypes of gastric cancer (intestinal type *vs* diffuse type), or sites of gastric cancer occurrence (antrum, corpus and cardia) between the gastric cancer and control subjects.

Shiotani *et al*[7] demonstrated that *H pylori*-positive patients with pruritic skin diseases may be at increased risk for the development of gastric cancer. Leung *et al*[8] reported the results of a randomized trial on *H pylori* eradication, demonstrating that *H pylori* infection ($OR = 2.13$), 45 years of age or greater ($OR = 1.92$), alcohol intake ($OR = 1.67$) and drinking water from a well ($OR = 1.74$) are risk factors that predict the progression of intestinal metaplasia as precancerous lesions.

Camargo *et al*[9] investigated the prevalence and the age of acquisition of *H pylori* infection in children aged 1 to 8 years old residing in Pasto, Colombia, which is associated with a high prevalence of gastric cancer as compared to those in children living in Tumaco, Colombia. They found that there were no significant differences between children in Pasto and Tumaco in the prevalence and age of acquisition of *H pylori* infection, suggesting that the risk for gastric cancer is associated with the bacterial virulence of the *H pylori* strains involved.

DYNAMICS OF GASTRIC MUCOSAL CHANGES

Kato *et al*[10] investigated the sex differences in mucosal response to *H pylori* infection and found that atrophy and intestinal metaplasia scores in the corpus with *H pylori* infection were more severe in men than in women, particularly in elderly patients. They also reported that while there were no differences between the sexes with regard to interleukin-8 mRNA induction, cyclooxygenase-2 (COX-2) mRNA expression was higher in men than that in women, concluding that these differences in response between men and women may account for the 2-fold difference in the incidence of gastric cancer between the sexes.

Shiotani *et al*[11] demonstrated that the gastric nitrate and pH levels were progressively increased from patients with histological normal diagnosis to those with antral-predominant gastritis to those with pangastritis, and then on to those with corpus-predominant gastritis, suggesting that the gastric pH level is one of the prognostic markers for corpus-dominant gastritis and probably for patients at high risk of developing gastric cancer.

Of the reports that addressed post-eradication changes in the mucosa, a study by Fichman *et al*[12] demonstrated that there was improvement in acute and chronic inflammation scores, the number of lymphoid follicles, as well as an increase in the number of gastric glands, while there was no change in intestinal metaplasia over time, during a mean follow-up of 23.15 mo after *H pylori* eradication in 89 patients. They further demonstrated that the Ki67 labeling index significantly decreased after eradication, while the MUC5AC and MUC6 expressions increased. Of the numerous reports published on post-eradication changes in the gastric mucosa, many reported improvement of intestinal metaplasia with eradication of *H pylori*, suggesting the potential role of *H pylori* eradication in the prophylaxis of gastric carcinogenesis[13-15].

Iijima *et al*[16] also reported that *H pylori* infection showed a stronger inhibitory effect on acid secretion in males than that in females. Ohata *et al*[17] demonstrated in a longitudinal cohort study in 4 655 healthy subjects with a mean follow-up of 7.7 years that 45 subjects subsequently developed gastric cancer. In this study, *H pylori* infection was established by serum-specific antibodies and the presence of chronic atrophic gastritis was confirmed by measurement of serum pepsinogen I and II. They also demonstrated that *H pylori* infection and gastric mucosal atrophy were both strongly implicated in gastric carcinogenesis and that *H pylori*-negative patients in whom there was no evidence of gastric atrophy did not develop

gastric cancer. Furthermore, they concluded that while *H pylori* infection is not directly associated with gastric carcinogenesis, it is indirectly implicated in it by promoting gastric atrophy, and severe gastritis with extensive intestinal metaplasia is a major risk factor for gastric cancer.

Suzuki *et al*[18] provided evidence that *H pylori* eradication exerted an inhibitory effect on the proliferation of epithelial cells as shown by the tissue content of hepatocyte growth factor (HGF) and Ki67 labeling index. Our previous study also demonstrated that *H pylori* eradication led to a decrease in the expressions of *p53* and multiple double minute 2 (MDM2) in the epithelial cells, which had been elevated prior to eradication[19] .

DNA DAMAGE

Ladeira *et al*[20], using the comet assay or single-cell gel electrophoresis, investigated the status of DNA damage in gastric epithelial cells from the antrum and corpus in *H pylori*-infected patients with gastritis of varying degrees, and demonstrated that the level of DNA damage in *H pylori*-infected individuals was significantly higher (8.4-fold) than that in non-infected individuals, with the levels of DNA damage significantly higher in those aged 50 years or more.

The DNA repair enzyme human oxoguanine glycosylase 1 (hOGG1) is known to be responsible for the repair of the 8-hydroxy-deoxyguanosine (8-OHdG) region. Of the hOGG1 polymorphisms identified, a Ser→ Cys polymorphism at position 326 was found to interact with atrophic gastritis, but not with antioxidant dietary or nutrient intakes, likely making patients with atrophic gastritis who also have the hOGG1 Cys allele more susceptible to gastric cancer[21] .

The concentrations of 8-OHdG, inducible nitric oxidative synthase (iNOS), nuclear factor κB (NF-κB), myeloid cell leukemia-1 (Mcl-1) and inhibitor of apoptosis protein (IAP) were reported to be significantly higher in patients with *H pylori* infection, in cancer tissues, and in stage 3 and 4 gastric cancer patients, pointing to the pivotal role that oxygen-free radical-mediated DNA damage due to *H pylori* infection plays in the development of gastric carcinoma from chronic gastritis[22].

VIRULENCE FACTORS

CagA proteins and gastric carcinoma

H pylori virulence factors include the cytotoxin-associated gene A antigen (CagA), the vacuolating cytotoxin (VacA), urease, and blood group antigen-binding adhesin (BabA). After the year 2000, much has been clarified about the behavior of CagA proteins, where *H pylori* could directly deliver the CagA protein into the host epithelial cell cytoplasm via the *cag* PAI-coded type IV export system[23]. Inside the epithelial cells, the CagA protein undergoes tyrosine phosphorylation by the host Src family protein tyrosine kinases, and the CagA protein binds an Src homology 2 (SH-2) domain-containing tyrosine phosphatase SHP-2, and stimulates the division and proliferation of gastric epithelial cells[24] .

Twenty percent of the tyrosine-phosphorylated CagA also binds carboxyl-terminal Src kinase (Csk) with 80% of the tyrosine-phosphorylated CagA binding SHP-2. The CagA-Csk interaction activates Csk and inactivates the Src family kinases, thereby bringing about a decrease in CagA tyrosine-phosphorylation as well as in CagA-SHP2 interactions as a feedback mechanism[25]. Through this mechanism, chronic infection with *cagA*-positive strains persists, thus causing the host damage.

There are two subtypes of the CagA proteins reported, namely the Western CagA and East Asian CagA[26] . Due to the presence of polymorphisms in tyrosine phosphorylation sites, the East Asian CagA proteins were reported to have a significantly higher SHP-2-binding affinity than the Western CagA proteins, whereas the Western CagA proteins with repeated EPIYA motifs are associated with greater CagA phosphorylation, and higher SHP-2-binding affinity, and greater induction of the hummingbird phenotype than the Western CagA proteins without repeated EPIYA motifs[26]. However, the East Asian CagA proteins, with a different amino acid sequence downstream of the third EPIYA motif, are reported to exhibit even greater activity than the potent Western CagA proteins.

Argent *et al*[27] also reported that the more EPIYA motifs present in the CagA proteins, the higher the degree of CagA tyrosine-phosphorylation and the level of CagA biologic activity in inducing human gastric epithelial (AGS) cell elongation (hummingbird phenotype), and the more likely they are to be associated with gastric cancer.

In the host cell, the tyrosine-phosphorylated CagA protein induces rearrangements of the actin cytoskeleton. The tyrosine-phosphosphorylated CagA inhibits the catalytic activity of Src family kinases and induces tyrosine-dephosphorylation of several host cell proteins, one of which has been identified as ezrin. Ezrin is a component of microvilli, and makes up the ezrin-radixin-moesin (ERM) family of proteins as a linker protein between actin filaments and membrane proteins, and thus is deemed responsible for the adhesion and division of molecules as well as for intracellular signaling. This suggests that ezrin dephosphorylation may be implicated in the mechanism of gastric carcinogenesis[28].

Given the well known fact that the incidence of gastric cancer in Okinawa Prefecture, Japan is quite low, Azuma *et al*[29] compared the *H pylori* strains prevalent in Fukui and Okinawa Prefectures, and found that, of the strains examined, those found in Fukui were all of the East Asian CagA subtype; and, of the 42 strains associated with gastritis in Okinawa, 6 strains (14.3%) were *cagA*-negative, 8 strains (19.0%) were of the Western CagA subtype, and 28 strains (66.7%) were of the East Asian CagA subtype; and all strains associated with gastric carcinoma were of the East Asian CagA subtype. The degree of inflammation, activity of gastritis, and gastric mucosal atrophy observed in the East Asian *cagA*-positive strains were significantly more advanced than that in the Western *cagA*-positive or -negative strains, suggesting that infection with East Asia *cagA*-positive *H pylori* strains is strongly associated with gastric atrophy and cancer.

Furthermore, Azuma *et al*[30] compared the distribution of CagA polymorphisms in the world and analyzed the

relationship between the CagA distribution and associated mortality rate of gastric cancer. In this report, they noted that no East Asian CagA proteins were found in the West, while all CagA proteins reported in Asia were of the East Asian CagA subtype, with the prevalence of the East Asian CagA subtype found to be correlated well with the mortality rate from gastric cancer, thus concluding that searching for CagA polymorphisms rather than mere *H pylori* infection may be of greater value in identifying patients at increased risk of developing gastric cancer.

Azuma *et al*[31] also reported on several genes in a pathogenicity island (PAI) known as *cag* PAI, of which the *cagA* gene is one. According to their report, the *cag* PAI can be classified by their polymorphisms into the Japanese and Western clusters, and their diversity is associated with the *vacA* and *cagA* genotypes; and all strains with the *s1c vaA* genotype as well as with the East Asian *cagA* genotype are found in the Japanese cluster. They also noted that patients infected with strains found in the Japanese cluster were associated with high grade gastric mucosal atrophy and that while nearly all patients with *H pylori* infection are found to be *cag* PAI-positive, of all *cag* PAI polymorphisms, the Japanese cluster-genotypes appear to be predominantly associated with advanced gastric atrophy and increased gastric cancer risk.

Kauser *et al*[32] investigated the chromosomal integrity or lack of the *cag* PAI across different populations by evaluating the biogeographical distribution of strains carrying the fully integral *cag* PAI. According to their report, the *cag* PAI was well conserved in the East Asian clinical isolates and most highly conserved in the Japanese clinical isolates, but least conserved in the majority of the European and African clinical isolates. They also found that the *cagE* and *cagT* genes were less often rearranged (18%) compared to the *cagA* gene, and the differing *cag* PAI rearrangement patterns were related to varying disease outcomes, with the *cagA* promoter and the left end of the *cag* PAI frequently rearranged or deleted in clinical isolates associated with severe pathology.

AI-Marhoon *et al*[33] proposed a model on the association between *cagA*-positive *H pylori* infection and distal gastric cancer, explaining how *cagA*-positive *H pylori* infection is associated with greater PGE_2 production, increased mucus thickness and hydrophobicity, which serve to protect and keep intact bacterial colonization and increase gastritis, and is therefore associated with the risk of distal gastric cancer.

Ladeira *et al*[34] showed that *cagA*, *vacA*, and *iceA* genotypes were associated with significant DNA damage in both the gastric antrum and corpus, indicating that they may serve as biomarkers for the risk of extensive DNA damage and possibly for gastric cancer.

Toward the end of 2003, Huang *et al*[35] performed a meta-analysis of the relationship between *cagA* seropositivity and gastric cancer in a total of 2284 patients with gastric cancer *versus* 2770 control subjects, demonstrating that *H pylori* infection and *cagA* positivity increased the risk for gastric cancer by 2.28-fold and 2.87-fold, respectively. They also reported that among *H pylori*-positive patients, *cagA* positivity further increased the risk for gastric cancer by 1.64-fold, and that for non-cardia gastric cancer by 2.01-fold, whereas the gastric cancer at the cardia was not associated with *H pylori* infection or *cagA* positivity, thereby concluding that *cagA* positivity is associated with a greater risk of developing gastric cancer than *H pylori* infection. Furthermore, Held *et al*[36] investigated the association between *cagA*-positive *versus cagA*-negative status and gastric cancer in a case-control study and found that *H pylori* antibody positivity was associated with a greater risk of gastric cancer than *H pylori* antibody negativity, and that cag*A* antibody positivity further increased the risk, while cag*A* antibody-negative *H pylori* infection was associated with a significantly greater risk for gastric cancer than *cagA* antibody- and *H pylori* antibody-negative status.

There are several reports, however, that tend to negate the role of *cagA* as a risk factor for gastric cancer, while acknowledging its importance. Hirata *et al*[37] reported on the functional variability of the *cagA* gene in 36 clinical strains isolated from Japanese patients with various gastric diseases. They demonstrated that while delivery of the CagA protein into the host epithelial cell cytoplasm in the majority of Japanese patients with *H pylori* infection is ensured via the type IV export system, and tyrosine-phosphorylation of the CagA protein is promoted, the intensity of CagA phosphorylation as well as the activation of the serum response element (SRE) vary greatly among the *cagA*-positive strains, with those associated with gastric cancer, MALToma and chronic atrophic gastritis found to be slightly higher than those associated with gastroduodenal ulcers. They also noted that while the *cagA* polymophisms and the number of EPIYA motifs repeated should account in part for the variety of resulting diseases, the association between *cagA* status and disease outcome might not be too strong.

Bachert *et al*[38] performed a functional analysis of the *cag* PAI in 25 *H pylori* strains each isolated from patients with gastritis, peptic ulcer disease, and gastric cancer, and demonstrated that *cagA* expression, translocation and tyrosine-phosphorylation were high but similar among all patient groups examined, suggesting that while the *cag* PAI-dependent processes remain an important *H pylori*-associated virulence factor, the determination of disease outcome may be highly complex and involve multiple bacterial and/or host factors. Similarly, Nishiya *et al*[39] investigated the role of the *cag* PAI by performing an analysis of 39 *H pylori* strains each isolated from Japanese patients with gastric cancer and chronic gastritis, respectively, where the presence of *cagA*, *cagE*, and *VirD4* homologue was studied. They reported that almost all patients were found to be *cagA*-positive, and there were no differences between patients with gastric cancer and chronic gastritis with regard to the expression of *cagA*, *cagE* and *VirD4*, thus concluding that the structure of the *cag* PAI might not be associated with the development of gastric cancer in Japanese patients.

Apart from this, while the interaction between *H pylori* infection and gastroesophageal reflux disease (GERD) has long been under debate, Ye *et al*[40] showed in a large population-based case-control study in Sweden that *H pylori* infection might prevent the development of GERD and reduce the risk of esophageal adenocarcinoma,

whereas gastric atrophy and infection with *cagA*-positive stains of *H pylori* might increase the risk for esophageal squamous cell carcinoma.

OTHER VIRULENCE FACTORS

In an analysis of gene expression profiles using gastric cancer cells, Yuan *et al*[41] reported that VacA affects cytoskeleton-associated genes, thereby disrupting cytoskeletal architecture; it also causes damage to cell cycle-related genes, and breaks the balance between cell proliferation and cell death, while at the same time inducing inflammatory responses.

In a comparison of the *hrgA* gene thought to encode a restriction enzyme in *H pylori* using clinical isolates from East Asian and Western countries, the prevalence of *hrgA*-positive strains was reported to be significantly higher in the Western countries (49%) compared to the East Asian countries (20%). However, the prevalence of the *hrgA* gene was not found to be related to disease specificity or to other important putative virulence factors (*i.e.,* CagA, VacA)[42].

Gastric mucosal interleukin 8 (IL-8) levels are related to the presence of both the *cag* PAI and the outer inflammatory protein (OipA)[43]. Yamaoka *et al*[44] examined the upstream IL-8 signal transduction pathway including the *H pylori*-associated IL-8 promoter and gene transcription region using *oipA*, *hepZ*, *cagE* gene knockout cultured cells. Maximization of *H pylori*-induced IL-8 gene transcription requires the presence of binding sites for ISRE-like element, NF-κB, and AP-1. Both the OipA and *cag* PAI are implicated in the process in which the interferon regulatory factor (IRF)-1 becomes activated by binding the interferon-stimulated responsive element (ISR)-like element, while the *cag* PAI, and not the OipA, is implicated in the activation of AP-1 and NF-κB. Again, the OipA, and not the *cag* PAI, is implicated *in vitro* and *in vivo* in the phophorylation of signal transducers and activators of transcription 1 (STAT1) as an upstream signal transduction pathway. Thus, while both the OipA and *cag* PAI are required for the full activation of the IL-8 promoter region, each acts via a different pathway that becomes distinct upstream of the IRF-1, with only the OipA implicated in the STAT1-IRF1-ISRE pathway. Gastric mucosal inflammatory processes are complex and involve different pathways centered on the IL-8 promoter region.

HOST FACTORS

In an experiment using gastric epithelial (AGS) cells, *H pylori* infection was found to increase the expression of plasminogen activator inhibitor (PAI)-2, suggesting that PAI-2 may play a role in influencing the progression to gastric cancer[45].

Nardone *et al*[46] demonstrated that in patients with up-regulation of COX-2 or microsomal PGE-synthase 1 (mPGES1) expressions by *H pylori* infection, the expressions of the anti-apoptosis marker *Bcl-x1* or the multi-drug resistance 1 gene (*MDR-1*) product P-gp were also increased, suggesting that this *H pylori*-induced process may contribute to gastric carcinogenesis and resistance to therapy.

It is reported that *H pylori* infection increases the mRNA expression of the apoptosis-regulating proteins Bid, Bax and Bcl-2[47], and that the tumor suppressor protein Bax translocates to the mitochondaria which subsequently undergoes fragmentation[48], suggesting that a loss of control over these apoptosis-regulating genes potentially leads to gastric carcinogenesis. Of note, these genes were found to be more markedly expressed in *cagA*-positive *H pylori* strains[47].

Of the IL-10 gene polymorphisms identified, the IL-10-1082G/-819C/-592C alleles (GCC haplotype) are reported to be associated with the highest mucosal IL-10 mRNA expression, and carriers of the GCC haplotype are associated with colonization by more virulent *cagA*, *vacAs1*, and *babA2*-positive *H pylori* strains[49], suggesting that cytokine gene polymorphisms may be implicated in the development of gastritis and gastric cancer.

The pro-inflammatory cytokine IL-1 is implicated in host susceptibility to *H pylori*-associated diseases, and recent studies have further suggested that this susceptibility may be under genetic control. Hartland *et al*[50] prospectively investigated the relationship between selected polymorphisms in three of the major IL-1 gene clusters for association with *H pylori* infection and/or gastric cancer, and reported a significant difference in the genotype frequencies for the *IL1R1 HinfI* SNP in those with current or previous evidence of *H pylori* infection as compared with those without (GG, 53% *vs* 75%; GA, 40% *vs* 19%; AA, 7% *vs* 6%; $P=0.0079$). They concluded that the relationship among IL-1 gene polymorphisms, *H pylori* and disease outcome is more complicated than previously proposed, and may not be associated with the development of gastric cancer. Polymorphisms of the IL-1 gene cluster (IL-1A, -B, and -RN) have been associated with advanced gastric cancer. In early-stage gastric cancer, Glas *et al*[51] investigated the role of host genetic susceptibility and concluded that the genotype *IL-1 RN*2/2* is associated with early-stage gastric cancer, where, in contrast to advanced disease, further pro-inflammatory cytokine polymorphisms are not implicated independently, but may act in combination to play a role in early stages of gastric carcinogenesis, while the role of *H pylori* infection in this process remains unclear. In addition, Chen *et al*[52] demonstrated in a case-control study of 142 gastric cancer patients and 164 control subjects that the carriage of *IL-1RN*2*, male gender, old age, and *H pylori* infection were associated with increased risk for gastric cancer, concluding that *H pylori* infection and *IL-1RN*2* are independent risk factors for gastric cancer, and *H pylori* testing and host genotyping may combine to help target the eradication of *H pylori* to high risk individuals.

Basak *et al*[53] investigated the role of *H pylori* lipopolysaccharide (LPS) in IL-1β gene expression and the signaling pathways leading to LPS-induced IL-1β secretion, and demonstrated that activation of the mitogen-activated protein (MAP) by LPS resulted in activation of the immediate downstream C/EBP β and the C/EBPβ-binding elements of the IL-1 promoter, thus leading to the expression of the IL-1β. Of note, they also reported that

LAP-induced activation of the Rac1/PAK1 signaling plays a role in activation of caspase-1 required for maturation of the pro-IL-1β.

Osawa *et al*[54] investigated the effect of *H pylori* infection on ghrelin, a novel peptide expressed in the gastrointestinal tract conferring potent protection against gastric mucosal inflammation[55], in 110 *H pylori*-positive and 50 *H pylori*-negative patients, and demonstrated that impaired gastric ghrelin production associated with atrophic gastritis induced by *H pylori* infection accounts for the decrease in plasma ghrelin concentration.

SOURCE OF GASTRIC MALIGNANCY

It is well accepted that epithelial cancer originates from transformation of tissue stem cells. However, this view was recently challenged by Houghton *et al*[56] in 2004, who proposed a new intriguing theory regarding gastric carcinogenesis. In their theory, it is advanced that bone marrow-derived cells (BMDC) may represent a potential source of malignancy, while they are usually recruited to sites of tissue injury and inflammation. In their experiments, C57BL/6 female mice were infected with *Helicobacter felis*, irradiated, and transplanted with galactosidase- and green fluorescent protein (GFP)-labeled bone marrow cells from male mice. At wk 20, the level of apoptosis was elevated in the infected mice, and galactosidase- and GFP-positive glands appeared. After 1 year of infection, these mice developed gastric carcinoma *in situ* or gastrointestinal intraepithelial neoplasms (GIN), which were found to be galactosidase- and GFP-positive. Houghton *et al*[56] noted that while acute injury or inflammation does not lead to a recruitment of BMDC, chronic infection of C57BL/6 mice with *Helicobacter* induces a repopulation of the stomach with BMDC, and these cells progress through metaplasia and dysplasia to intraepithelial cancer. This new hypothesis appears to have far-reaching implications for future research in gastric carcinogenesis.

H PYLORI ERADICATION AND GASTRIC CANCER

In the current review, we have addressed issues surrounding *H pylori* infection and gastric carcinogenesis. Not only long-term morphological but also genetic studies will be required to clarify whether or not improvement in gastric atrophy and intestinal metaplasia after *H pylori* eradication would lead to prophylaxis of gastric cancer or when would be the "point of no return" for such prophylaxis in the course of patient follow-up. Further long-term research into the morphological changes associated with *H pylori* eradication is also required to explore strategies for the prevention of gastric cancer with *H pylori* eradication.

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REVIEW

A concomitant review of the effects of diabetes mellitus and hypothyroidism in wound healing

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Abstract

This paper reviews the negative impact of diabetes mellitus or hypothyroidism on wound healing, both in experimental and clinical settings. Since both are metabolic disorders of great clinical importance, special attention is given, not only to their pathophysiology, but also to their biochemical and histological effects on tissue integrity and regeneration. Also, special focus is awarded on wound healing of the gastrointestinal tract, i.e. in intestinal anastomosis, and how these disorders can lead to wound dehiscence. Since diabetes mellitus and hypothyroidism can coexist in clinical settings, more research must be directed on their influence on wound healing, considering them as one clinical entity.

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Key words: Diabetes mellitus; Hypothyroidism; Wound healing; Anastomosis

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INTRODUCTION

Wound healing is an integral part of recovery of critically ill patients[1]. They often are at risk for impaired healing because of their compromised body systems and multiple risk factors. Disruption of healing may result in delayed healing, dehiscence, infection, and death. Understanding healing and the factors that may impede it provides the physician with the foundation on which care is planned to facilitate wound healing.

IMPEDIMENTS TO WOUND HEALING

The treatment of chronic wounds presents a challenge to physicians, caregivers, and patients[2]. A familiarity with the factors that impede healing is necessary if treatment of chronic wounds is to be effective. These wounds often heal in a short period of time if the factors inhibiting wound healing are adequately identified and managed. Recombinant growth factor therapy may provide an additional stimulus to healing in certain types of chronic wounds. However, there remains no substitute for a physiologic environment conductive to tissue repair and regeneration, without which the efficacy of growth factor therapy is questionable. Some of the most commonly encountered and clinically significant impediments to wound healing include tissue hypoxia, infection, presence of debris and necrotic tissue, use of anti-inflammatory medications, a diet deficient in vitamins or minerals, or general nutritional deficiencies, tumors, environmental factors, and metabolic disorders such as diabetes mellitus. Treatment of chronic wounds should be directed against the main etiologic factors responsible for the wound. Moreover, factors that may impede healing must be identified and corrected for healing to occur if possible.

Especially, regarding diabetes mellitus, there have been numerous experimental and clinical studies, not only establishing its negative impact on wound restoration but also studying its metabolic pathophysiology in comparison with every stage of wound healing. This is not only because diabetes mellitus is one of the most common clinical entities found, but also because it coexists with other metabolic disorders, such as hypothyroidism. While hypothyroidism alone, or as part of a clinical condition, like myxedema, is a well established disease, little research has been made to determine its exact effect on tissue healing and the biomechanical profile of wounds in hypothyroid patients.

COMPLICATIONS OF DIABETES MELLITUS

The long term complications of both types of diabetes mellitus are usually categorized into macrovascular, microvascular, and neuropathic diseases[3]. Macrovascular complications are associated with an accelerated rate of atherosclerosis and an increased propensity for peripheral vascular disease, myocardial infarction, and cerebrovascular accidents. Microvascular complications include retinopathy

and nephropathy and are associated with thickening of the capillary basement membrane. Also, the current suggestion by investigators states that microcirculatory damage is apparent in the skin and subcutaneous tissue, leading to impaired wound healing. An intact microcirculation is required for tissue nutrition, removal of waste products, inflammatory responses and temperature regulation therefore, logically any defect in microvascular function adversely affects tissue repair. One undisputed structural change that occurs in people with diabetes is thickening of the basement membrane, the extracellular matrix (ECM) bellow the cells lining the vessels. This is a consistent finding in the capillaries and arterioles of people with diabetes[4]. Studies have demonstrated that in early diabetes, microvascular blood flow is increased with prolonged exposure to hyperglycemia[5]. This is explained by increased production of nitric oxide by the endothelial cells as a result of vessel wall damage and heightened viscosity of the blood due to glycosylation of hemoglobin[6]. Hyperemia and capillary hypertension ensue. Precapillary resistance is reduced and further vasodilatation occurs in response to increased metabolic demand and oxygen requirements of damaged tissue. In turn, shear stress on the vascular wall increases leading to accumulation of ECM and the protein fibronectin in response to this chronic injury. Further basement membrane thickening occurs, altering membrane charge and as a result affecting capillary permeability. Microangiopathy-capillary disease has progressive pathology, particularly affecting cells in which glucose transport and metabolism are independent of insulin, such as endothelial cells[7]. Persistent hyperglycemia encourages the conversion within the endothelial cells of glucose to sorbitol. Cellular edema occurs, as sorbitol cannot diffuse across the cell membrane. This results in metabolic alterations, membrane function alteration and basement membrane thickening. It is also thought that hyperglycemia leads to increased protein synthesis, deposition of abnormal ECM proteins and further basement membrane thickening. However, there is a lack of human experimental evidence to support this[6]. As microvascular disease progresses usually in parallel with the duration of diabetes, impaired vascular reactivity and limited hyperemia result in the loss of normal autoregulatory function[4]. Homeostatic balance relies on the microvascular circulation maintaining a constant blood supply despite fluctuations in perfusion pressure. However, a thickened and rigid basement membrane restricts the normal hyperemic response to tissue damage and hypoxia ensues. Skin tissue breakdown inevitably occurs.

Neuropathic complications include damage to sensory, motor, and autonomic nerves.

WHY BLOOD GLUCOSE AFFECTS HEALING

Patients with diabetes often have wounds that are difficult to heal[8]. The initial barrier to healing is an increased blood glucose level, which causes the cell walls to become rigid, impairing blood flow through the critical small vessels at the wound surface and impeding red blood cell permeability and flow. Impairment release of oxygen by hemoglobin results in oxygen and nutrient deficits in the wound. A less optimal immune function also contributes to poor wound healing in patients with diabetes. When blood glucose levels are persistently elevated, chemotaxis and phagocytosis are compromised. Chemotaxis is the process by which white cells are attracted to the site of infection, while phagocytosis is the ingestion of bacteria by white cells. Both processes are important in controlling wound infections. Diabetic infections take a longer time to heal because of delayed macrophage introduction and diminished leukocyte migration, which causes a prolonged inflammatory phase in the wound healing cascade.

Protein-calorie malnutrition and the resultant body composition changes are an additional consideration in wound healing. Patients with diabetes often have a progressive loss of lean body mass, which is replaced with a metabolically inactive fat mass.

The absence or deficiency of insulin in diabetes mellitus causes impaired metabolism of carbohydrates, fat and proteins, which are necessary for cellular activities and tissue synthesis in wound healing[9]. Insulin is required for glucose to enter cells as to provide a source of energy for uptake of amino acids to synthesize proteins and for inhibition of adipose tissue lipolysis. Glucose is also needed to supply energy for fibroblastic and polymorphonuclear (PMN) activities during wound healing. Altered glucose metabolism, as seen in diabetes mellitus, leads to defective metabolism of these nutrients and reduces fibroblastic/PMN activity, causing impaired wound healing.

Altered protein metabolism

Protein is essential for the synthesis of collagen structures that establish wound tensile strength. Without adequate tensile strength of collagen structures, dehiscence and evisceration can occur. Protein deficiencies also impair formation of new capillaries, fibroblastic activity, and the bactericidal activity of PMNs. Insulin, which is lacking in diabetes, is the anabolic hormone that stimulates protein synthesis, so to promote wound healing, both insulin and protein need to be provided in adequate amounts for protein tissue rebuilding.

Altered lipid metabolism

Without insulin and glucose for energy, fat is the primary source of body fuel. So, when fatty acids, which are used to form cell membranes, are used for energy, as in poorly controlled diabetes mellitus, healing is impaired, since there is delayed cell membrane synthesis.

Decreased chemotaxis, phagocytosis, bacterial killing[10,11], decreased heat shock protein expression[12], less antioxidant synthesis , and increased oxygen free radical generation[13,14] during the early phase of wounding healing all have been implicated in impaired diabetic healing. Additionally, growth factor depletion[15,16], increased glucocorticoid concentration[17], decreased cell proliferation[18], and up-regulation of apoptosis[19] characterize the later phases of diabetic healing resulting in poorer granulation tissue formation. Diabetes mellitus lowers extracellular matrix synthesis following wounding[20].

While the relation of diabetes mellitus to wound healing is well elicited in numerous other studies and

papers[21-25] demonstrating that it is a complex metabolic disorder whose components have several direct and indirect effects on the healing of wounds, Komesu *et al*[26] evaluated the healing process during early phases of experimental diabetes on rat skin and found that not only the initial healing phase has a slow beginning and tends to last longer, but also that healing areas show lower density of neutrophils after surgery, and in addition, after three days, when the neutrophils leave the healing area and are replaced by macrophages, diabetic animals show a higher number of neutrophils. The principal conclusion is that although diabetes is a chronic progressive disease, acute diabetes can be associated to subclinical alteration, and is considered responsible for deficiencies in defense cells and in repair tissue failures.

METABOLIC PATHOPHYSIOLOGY OF DIABETES MELLITUS

There are three possible ways that hyperglycemia contributes to the metabolic pathophysiology of diabetes-related complications[27].

The first hypothesis is that abnormal glucose levels alter the actual control of cellular Na^+/K^+ ATPase activity[28]. Hyperglycemia leads to increased polyol pathway activity, which leads to a depletion of myo-inositol stores. This involves the biochemical pathway in which glucose is converted to sorbitol by aldose reductase. Because the conversion of sorbitol to fructose (by sorbitol dehydrogenase) is slow, sorbitol tends to collect in cells. The end-product of the sorbitol to fructose is NADH. The increased conversion to fructose leads to decreased NADPH and increased risk for oxidative stress. The increased levels of sorbitol may increase the osmotic load in the cells. Sorbitol inhibits myo-inositol uptake, which in turn leads to the alteration in Na^+/K^+ ATPase activity.

A second metabolic abnormality that results from hyperglycemia may be related to the activity of protein kinase C (PKC). Diabetes appears to increase the synthesis of diacyl-glycerol, which in turn leads to increased PKC activity. PKC is a key signaling receptor for many cellular activities including proliferation, contraction, calcium influx and others.

Thirdly, hyperglycemia also leads to the production of pathologic by-products. Hyperglycemia leads to 'advanced glycosylation end products' (AGEs), which are large aggregates of aldoses covalently bound to reactive amino groups. AGEs may lead to increased oxidative stress and activate a key transcription factor, nuclear factor (NF)-κB. NF-κB is involved in many cytokine-related cell responses. For instance, AGEs appear to induce the production of platelet-derived growth factor (PDGF), tumor necrosis factor-α, and interleukin-1α. AGES may also lead to collagen cross-linking and inhibit normal collagen degradation.

Fahey *et al*[29] examined leukocyte infiltration and the appearance of tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) in wound chambers implanted in normal and streptozotocin-induced diabetic mice. The data suggest that delayed healing on diabetes is associated with altered leukocyte infiltration and wound fluid IL-6 levels during the late inflammatory phases of wound healing. These observations suggest that while the initial inflammatory response is intact, diabetes can impair the late inflammatory response to wound healing, data that corroborate previous reports from Goodson and Hunt[30-32] that implicate a defective inflammatory response to wounds in the pathophysiology of delayed wound healing are associated with diabetes. They have shown that obesity, insulin resistance, hyperglycemia, and depressed leukocyte function interfere with collagen synthesis and thus impair wound healing. Their studies of skin and subcutaneous wounds in diabetic mice showed that the granulocyte influx is slow and is associated with depressed synthesis of protocollagen and collagen. Hennessey *et al*[33] postulated that altered collagen metabolism seen in experimental diabetes could be related to the presence of increased tissue levels of advanced glycosylation end products. Others, like McMurry[34] suggested that impaired leukocyte function may contribute to the impaired healing in diabetes based on abundant evidence that neutrophil chemotaxis and phogocytosis are inhibited by hyperglycemia and poorly-controlled diabetes mellitus[35]. Robson *et al*[36] have addressed the issue of wound infections in diabetic patients by evaluating the relative growth of various bacteria in the presence of hyperglycemia, and found that gram-positive bacteria thrive in hyperglycemic serum and that Gram-negative bacteria grow less well in hyperglycemic serum, which could partially explain the clinical observation that diabetic patients are prone to staphylococcal infection.

Additionally, defects in wound healing of experimental diabetes may be corrected, at least in part, by administration of large amounts of vitamin A[37] or zinc[38].

Last but not least, uremia is another factor that alters healing[39]. Patients with diabetes mellitus are prone to silent or overt renal disease. Increased urinary protein loss in these diseases predisposes the patient to edema, which contributes to impaired tissue repair. In addition, uremia is another factor that independently contributes to the altered healing.

WOUND HEALING AND GROWTH FACTORS

Recent advances in wound treatment include topical growth factor therapy, which has been successful in diabetic wounds. Platelet-derived growth factor (PDGF), fibroblast growth factor, and epidermal growth factor have been shown to accelerate tissue repair in an experimental wound model. Insulin is one of the primary anabolic hormones in the body, and numerous studies have shown beneficial effects of insulin therapy on wound healing. Insulin increases wound tensile strength and stimulates protein anabolism in skin and muscle. One possible reason for their success is the relative deficiency of growth factors in chronic wound fluid due to decreased supply, increased binding, or increased degradation of the naturally occurring growth factors. A study[40] was performed to examine the level of the insulin-degrading enzyme (IDE) in diabetic wound fluid and to relate this to glucose control [hemoglobin A_{1c} (HbA_{1c}) levels]. An excess of this enzyme could contribute to reduced levels of growth factors in

wound fluid. The authors showed that insulin-degrading activity is present in human wound fluid, and that the activity is higher in subjects with diabetes. The biochemical properties of the wound fluid insulin-degrading activity are consistent with the properties of the insulin-degrading enzyme (insulysin). Insulin-degrading activity correlates with HbA_{1c} levels, suggesting a mechanism for the relationship between glucose controlling and wound healing. Improvement in glucose control is a critical factor in wound healing, but a reduction in wound fluid insulin-degrading activity is also a potential therapeutic approach.

PDGF levels have been examined by radioimmunoassay in wound tissue of normal and diabetic rats, while immunohistochemical analysis can be utilized to localize and characterize PDGF immunopositive cells at the wound site of normal and diabetic animals[41]. The findings suggest that absence of an initial increase in PDGF may play an important role in poor wound healing observed in diabetic animals, and that the reduction in PDGF could be related to decreased cellular PDGF production rather than lack of PDGF-producing cells. The authors concluded that perhaps the diabetic state inhibits cellular PDGF gene expression signaled by wounding or interferes with normal PDGF expression at the wound site.

To examine the effects of recombinant growth factors *in vivo*, impaired wound healing has been studied in genetically diabetic C57BL/KsJ-db/db mice[42]. Large full-thickness skin wounds, made on the backs of these mice, exhibited significant delays in the entry of inflammatory cells into the wound, the formation of granulation tissue, and in wound closure, when compared to their nondiabetic littermates. Recombinant human platelet-derived growth factor (rPDGF-BB), recombinant human basic fibroblast growth factor (rbFGF), or combinations of both were applied topically to the wounds for five to fourteen days after wounding. Combinations of rPDGF-BB and rbFGF improved all parameters of healing but not to a greater extent than either growth factor alone.

Numerous other studies have held to elucidate the effect of growth factors in the process of healing of diabetic wounds. Hennessey *et al*[43] studied the interactions of topically applied insulin and epidermal growth factor (EGF) in diabetic rats. The EGF and insulin promoted a 202% increase over controls in collagen synthesis after fifteen days, while diabetic rats receiving EGF or insulin alone had significantly less collagen than controls. The individual effects of insulin and EGF added synergistically for a net gain in wound collagen content are a gain that has not been observed with either EGF or insulin alone. Bitar *et al*[44] have proved that diabetes induces not only a marked reduction in insulin-like growth factor-I (IGF-I) levels both in wound fluid and serum of diabetic rats, but also a reduction in the levels of IGF binding proteins and transforming growth factor-β (TGF-β) in diabetic wound fluid. The investigators reported that the diabetic wound fluid increased levels of extracellular matrix gelatinases, and that a single dose of TGF-β partially reversed the diabetes-related decrease in the tensile strength of standardized incisions.

Furthermore, elevated levels of glucose have been shown to affect insulin signaling in various ways. Hyperglycemia can alter glucose-induced insulin secretion, a phenomenon referred to as glucose toxicity. Glucose is known to affect insulin action as well by regulating the expression of several genes, including the IGF-I receptor (IGFR) and insulin receptor (IR) genes, at both the transcriptional and translational levels[45]. Moreover, hyperglycemia can inhibit insulin action. This inhibition is thought to be a result of serine phosphorylation through a PKC-mediated mechanism as well as by activation of protein tyrosine phosphatases, which deactivates the IR function. In addition to its possible involvement in inducing complications of chronic diabetes, glucose down-regulates its own transport and metabolism. In this study, the authors investigated the relative roles of hyperglycemia, insulin, and IGF-I, all of which are abnormal in diabetes, in primary murine skin keratinocytes. The results showed that in the presence of high glucose (20 mmol/L), the glucose transport rate of primary proliferating or differentiating keratinocytes was downregulated, whereas at 2 mmol/L glucose the transport rate was increased. These changes are associated with changes in the GLUT1 expression and in the affinity constant (km) of the transport. Exposure to high glucose is associated with changes in cellular morphology, decreased proliferation and enhancement of Ca-induced differentiation of keratinocytes. Furthermore, in the presence of high glucose, ligand-induced IGF-I receptor, but not insulin receptor (IR) autophosphorylation, is decreased. Consequently, in high glucose, the effects of IGF-I on glucose uptake and keratinocyte proliferation are inhibited. Interestingly, lack of IR expression in IR-null keratinocytes abolishes insulin-induced glucose uptake and partially decreases insulin- and IGF-I-induced proliferation, demonstrating the direct involvement of the IR in these processes. The results demonstrate that hyperglycemia and impaired insulin signaling might be directly involved in the development of chronic complications of diabetes, like wound healing, by impairing glucose utilization of skin keratinocytes as well as skin proliferation and differentiation.

CONFLICTING RESULTS

In a recent retrospective study though[46] that was designed to assess the contribution of several factors that could be incriminated in the occurrence of abdominal wound dehiscence in major abdominal operations, diabetes mellitus was found not to be a significant risk factor, in contrast with advanced age (> 65 years), emergency operation, cancer, haemodynamic instability, intra-abdominal sepsis, wound infection, hypoalbuminemia, ascites, obesity, and steroids. The mortality and the possibility of dehiscence seem to correlate directly with the number of those risk factors.

HISTOLOGICAL, HISTOCHEMICAL AND BIOCHEMICAL STUDY OF DIABETIC WOUNDS

Prakash et al[47,48] have studied surgical wounds in albino rats. Uncontrolled diabetics, controlled diabetics and

normal hypoglycemic rats were used for study and compared with normal control animals. The parameters of histology, histochemistry and biochemistry were assessed. Healing was delayed in the uncontrolled diabetic group and was significantly ameliorated by the administration of insulin, but not to an extent as to be better than normal controls. More specifically, the uncontrolled diabetic group showed a more marked and prolonged inflammatory response. The first appearance of scar was after seven days as contrasted at five days in the other groups, while at the end of fifteen days the scar was significantly more vascular and cellular in the uncontrolled diabetic rats. Histochemically, the precollagen (reticulin) fibers were present in moderate amounts on the 5th post-operative d, increasing up to the 10th d, after which a decline was obvious. As a contrast, practically no stainable reticulin was seen on the 5th d in the diabetic group. It appeared on the 7th d and increased till the 15th d. The reticulin fibers in that group were sparse and poorly staining. The uncontrolled diabetic group showed delay in the appearance of collagen, increasing up to the 15th d, when it was less deeply staining and less dense, in contrast with the other groups where collagen was present in minimal amounts on the 5th d. The acid and neutral mucopolysaccharides were also under study. Traces were present on the second day after which there was an increase till the seventh day. A decline in stain of this substance was present thereafter. This response was observed in the other three groups. The uncontrolled diabetic group differed by showing the presence of mucopolysaccharides till the 15th d, indicating activity of the healing processes. The hydroxyproline content was seen to register a fall on the second day after wounding as compared with the first day. Subsequently, a steady rise was seen up to the 7th d. In the uncontrolled diabetic rat group there was a rise obvious from the 5th d onwards after which there was a steady gain in hydroxyproline content, to values lower than in the other three groups.

INFLUENCE OF DIABETES ON GI WOUND HEALING

While it is widely accepted that changes in the extracellular matrix, particularly due to collagen metabolism and related disturbances like diabetes mellitus, are a prognostic factor for anastomotic leakages[49,50], Gottrup *et al*[51] have investigated the influence of experimental diabetes on the healing of incisional wounds in stomach and duodenum. Experimental diabetes impaired the mechanical strength of healing wounds in stomach and duodenum. The reduction was most pronounced for breaking energy, while only duodenum showed a decrease for breaking strength of the tissues, an impairment that became more pronounced by increasing healing time. The investigators showed that there is a relation between mechanical strength and total collagen content and that insulin treatment prevents these retardations.

The influence of diabetes on the healing of HCL-induced gastric lesions and the healing promoting effect of basic fibroblast growth factor (bFGF) on these lesions under diabetic conditions induced in rats by streptozotocin has been reported[52]. Diabetic conditions could not affect the development of HCI-induced gastric lesions but could significantly delay the healing of these lesions. Daily administration of insulin could return high blood glucose levels to normal ranges and significantly antagonize the delayed healing of these lesions. The delayed healing in diabetic rats could also be significantly promoted by recombinant human basic fibroblast growth factor without any effect on blood glucose level. The mucosal bFGF levels in streptozotocin-diabetic rats are significantly lower under basal conditions before HCI treatment and do not increase after injury, yet such dysregulation of bFGF production could be partially restored by insulin treatment. These results suggest that diabetic conditions have deleterious influences on the healing of acute gastric lesions in both an insulin- and bFGF-sensitive manner, and that the administration of exogenous bFGF antagonizes the delayed healing of gastric lesions observed in diabetic animals.

Verhofstad and Hendriks[53] demonstrated in an experimental study in rats that diabetes indeed lower bursting strength in both ileal and colon anastomosis. However, this effect is not secondary to decreased collagen deposition bur rather due to increased abscess formation around the anastomosis. Incubation of fibroblasts isolated from ileum or skin of nondiabetic rats with diabetic and nondiabetic serum only affects collagen synthesis in skin fibroblasts, suggesting that fibroblasts from different organs are distinctly affected by diabetes[54]. Postoperative restoration of blood glucose by insulin[55] or islet transplantation[56] only partially restores bursting pressure. Enhanced matrix metalloproteinase activity has been shown to be present in diabetic colon anastomosis, possibly explaining the weaker breaking strength. In contrast to dermal healing, diabetic anastomoses are characterized by an elevated number of neutrophils, which could serve as a source of matrix metalloproteinases[57].

Martens *et al*[58], studying the postoperative changes in collagen synthesis in intestinal anastomosis of the rat, investigated the differences between small and large bowel. The results showed that the ileum responds more quickly and strongly to wounding than the colon, at least as far as the production of new collagen is concerned. Possibly, this phenomenon contributes to the lower failure rate apparent for anastomoses in the small bowel.

Verhofstad *et al*[59] have studied the cellular and architectural parameters of anastomotic healing, this time in diabetic rats. The results showed that although anastomotic necrosis, edema, and epithelial recovery are not affected by diabetes, in diabetic rats, the number of polymorphonuclear cells and macrophages is significantly increased, both in ileal and colonic anastomosis. Repair of the submucosal-muscular layer in colonic anastomoses from diabetic rats is impaired, but in ileal anastomoses no difference is found. In the anastomotic area, collagen deposition remains unaffected by diabetes, thus proving that experimental diabetes leads to alterations in cellular components involved in the early phase of repair of intestinal anastomoses, but not to a reduced accumulation of wound collagen.

Most recently, Onodera *et al*[60] studied anastomotic

leakage in diabetic rats. The purpose of the study was to determine whether collagen synthesis was correlated with the anastomotic strength in diabetic animals in special reference to collagen type differences. The investigators showed that although there is no statistical significance between the control and diabetic groups in hydroxyproline concentrations and that although the expressions of mRNA of collagen type III in both groups are not statistically different, the expression of collagen type I in the diabetes group merely increases and is clearly less than that of the control group. The increase of type I collagen is much more pronounced and long-lasting than that of type III collagen. Brasken *et al*[61] suggested that type I collagen synthesis is enhanced for at least 2 wk after anastomosis whereas type III collagen synthesis is increased only during the first week. Taken together with previous experimental results, the weakness of anastomotic healing with diabetic rats is not attributed to the total contents of collagen but to changes of newly formed collagen, i.e. mainly the blockage of the type I collagen synthesis.

CONFLICTING RESULTS

Although, other investigators like Witte and Barbul[62] and Wagner and Egger[50] have recognized that colon healing is a structured cascade of different phases that can be affected by a multitude of local (infection, ischemia) and systemic (diabetes, malnutrition, anemia, hypothermia, trauma) factors, a recent retrospective study done to identify the risk factors for anastomotic leakage after left-sided colorectal resections with rectal anastomosis, showed that diabetes is a non-significant variable[63].

HYPOTHYROIDISM AND WOUND HEALING

Hypothyroid patients have long been thought to have a higher risk of surgical morbidity and mortality[64,65]. Thyroid hormone deficiency does cause derangements of cardiovascular[66], pulmonary[67], renal[68] and central nervous system functions[69], and alters drug metabolism[70] in ways that could predispose to surgical complications. Surgery in hypothyroid patients is associated with an increased risk of minor perioperative complications, which should be anticipated and preemptively managed in the course of their anesthetic and surgical care[71], a conclusion which was validated by Ladenson PW *et al*[72], who reported that postoperatively, hypothyroid patients more commonly have, among others, gastrointestinal and neuropsychiatric complications than control patients.

Since collagen is the only protein in the body containing hydroxyproline in significant amounts, urinary excretion of hydroxyproline has been considered an index of collagen metabolism since the 60's[73]. Hormones concerned with body growth and protein metabolism particularly affect collagen metabolism[74]. Thyroid hormone can stimulate hydroxyproline excretion and correct the reduction of hydroxyproline excretion in hypothyroidism[75].

Kowalewski and Yong[76] have shown a significant increase of a saline-extractable, and total soluble hydroxyproline, and a reduction of insoluble fraction in the normal wound. This might be due to vigorous biosynthesis of collagen. In contrast, no evidence of the synthesis of new collagen has been found in the wounds of hypothyroid rats. Insoluble fractions are higher in the wounds of hypothyroid rats than in normal control animals, suggesting that biosynthesis, solubility, and overall metabolism of collagen in hypothyroid rats are deficient.

Experimental work by Lennox and Johnston[77] has shown that wound healing is accelerated by a mean of 2.5 d in a hyperthyroid group of rats and delayed by a mean of 2.0 d in the hypothyroid group as compared with control rats. Mehregan and Zamick[78] demonstrated that T_3 has a beneficial effect on the healing of deep dermal burns in rats. There is better organization of collagen bundles, fewer retraction spaces, and smoother scars. Another experimental study by Herndon *et al*[79] showed that levels of thyroid hormone have a profound effect on the rate of healing of burn wounds in thyroidectomized guinea pigs. They showed that epithelization is inhibited at low and high doses of levothyroxine sodium. However, administration of levothyroxine sodium at the intermediate dose of 30 μg/kg results in improved wound closure relative to euthyroid control subjects. This finding is consistent with the results of Kivirikko *et al*[80], who showed that the rate of collagen synthesis is decreased both in hyperthyroidism and hypothyroidism**.** On the other hand, there are clinical reports of kelloid development during the healing process of wounds of patients with hyperthyroidism[81], but no change in wound healing with euthyroid hamsters receiving ip T_4[82]. A retrospective study[83] concluded that the conditions of patients with laryngeal tumors treated with surgery or surgery and radiation should be evaluated for hypothyroidism. When patients become hypothyroid, they should be treated promptly. Treatment of thyroid dysfunction can accelerate healing in patients with complications such as flap edema, flap necrosis, or fistula. Cannon[84] reported in an experimental study that although hypothyroidism alone, occurring in patients undergoing treatment for head and neck neoplasms, has no significant unfavorable impact on wound tensile strengths or flap survival, when combined with preoperative radiation, there are statistically deleterious effects on both wound tensile strengths and flap survival. Histologically, collagen fibers within the wound appear shorter and thinner, which probably account for decreased wound tensile strengths. It is recommended that thyroid function tests should be done routinely before and after therapy in all patients with head and neck cancer undergoing combined surgery and radiation.

Another study also demonstrated that the hormonal replacement therapy in hypothyroidism cases is beneficial with regard to wound healing and the results are more satisfactory if zinc is added to the therapy[85]. A decrease in serum zinc level in hypothyroidism has been reported in many clinical and experimental studies[86]. As noted above, experimental and clinical studies on hypothyroidism have shown that L-thyroxine replacement therapy has a positive effect on wound healing, which is generally delayed in this disorder. Elevation of wound breaking strength is one of the most important phenomena observed during the wound healing. In addition, the important factors

for the increase of breaking strength are the amount of collagen of the wound and the increase in intramolecular-intermolecular cohesive bounds of collagen present in the wound (cross-linking), the later being most important[84]. Lysyl oxidase, a copper-dependent enzyme, is responsible for the development of covalent bounds during collagen aggregation, in the activation process of which, it was recently shown that zinc has an important function[87]. However, there are some studies suggesting that zinc has no effect on the amount of collagen synthesis during wound healing[88]. Zinc given alone to the hypothyroid animals provides a small rise in tissue hydroxyproline level (not significant). These findings, consistent with the current recognition of collagen metabolism mentioned above, point out that zinc probably does not have a direct effect on the amount of collagen synthesis, but rather affects more directly the cross-linking of formed collagen.

Natori *et al*[89], in order to analyze the relationship between hypothyroidism and wound healing, induced a state of severe hypothyroidism in rats by surgically resecting their thyroid glands, and then assaying the levels of hydroxyproline and procollagen peptide (types I and III), which are the precursors of collagen (type IV: 7S) in wounds. The results of the assay of collagen and hydroxyproline in wounds indicate a significant decrease in type IV collagen and hydroxyproline in the surgical hypothyroidism rat group during the inflammatory phase and extending to the proliferative phase. These findings suggest that thyroid hormone is associated with the proliferation and secretion of fibroblasts in the process of wound healing. In the state of hypothyroidism, it appears that the suppression of thyroid hormone secretion causes a disturbance in the metabolical activation in tissues and the synthesis of collagen extending from the inflammatory phase to the proliferative phase.

In vitro, keratinocyte proliferation is retarded in T_3-deficient medium relative to T_3-replete medium[90]. *In vivo*, topical T_3 stimulates epidermal proliferation[90,91] and topical triac, the mild TH analog, thickens skin[92]. The keratin genes encode the intermediate filaments, making about 30% of the protein of the epidermis. Some associations between the keratin genes and specific phases of skin growth have been made[93,94]. Fox example, K6a, K16, and K17 are associated with epidermal proliferation and wound repair. In K6a knockout mice[95], the absence of K6a results in diminished superficial wound healing, but no change in full thickness wound healing. When K16 is over-expressed in cultured human keratinocytes, proliferation is enhanced[96]. However, human K16 overexpressed in mice results in delayed wound healing in the transgenic animals[97]. *Ex vivo* investigation suggested that the K16 overexpression inhibits keratinocyte migration. Although TH stimulates expression of K6a both *in vivo* and *in vitro*[98], only negative TH response elements have been identified for the keratin genes associated with proliferation[99].

The following investigation has been undertaken to ascertain the need for TH in optimal wound healing and to clarify the effect of TH on the expression of wound healing-associated keratin genes[98]. The study showed that K6a, K16, and K17 gene expressions are decreased in T_3 deficient keratinocytes. To determine whether the *in vitro* T_3-mediated keratin gene expression could be reproduced *in vivo*, and whether that pattern is associated with significantly poorer wound healing, the investigators contrasted the impact of surgical hypothyroidism on wound healing and proliferation-associated keratin gene expression. The result showed that hypothyroid mice have indeed retarded wound healing, suggesting that T_3 is necessary for optimal wound healing. T_3 exerts its influence by stimulating the proliferation of cytokeratins 6a, 16, and 17.

CONCLUSION

It is well established that both diabetes mellitus and hypothyroidism are considered to have a negative impact on wound healing, not only as part of complex metabolic disorders, but also as clinical entities. Especially for the diabetic part, numerous studies have been undertaken, not only to provide explanations in regards to its metabolic pathophysiology, but also to provide evidence for its effect on wound integrity and dehiscence. As for the hypothyroid status and its exact role in wound healing, much more studies are still needed, so as to enlighten its close relationship with collagen, and therefore wound leakage or not, but also as clinical entities, as previous studies of impaired blood flow and its relationship to wound healing, using various suturing techniques[100], and of the evaluation of adhesion formation among different types of intraperitoneal catheters used in surgical practice[101], have proven.

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REVIEW

Is interferon-beta an alternative treatment for chronic hepatitis C?

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Abstract

The treatment of chronic hepatitis C (CHC) is still far from optimal, particularly for those subpopulations that do not respond to the standard combination therapy with Interferon-α (IFNα) plus ribavirin. Although in some cases the use of higher doses or longer treatment periods may be effective, these approaches are generally associated with a higher incidence of adverse events, which may either lead to a reduction in patient compliance or require drug withdrawal. IFNβ could represent an interesting alternative for treating CHC patients. Controversial data about IFNβ efficacy in CHC exist, the main reason being that many results stem from pilot studies with small cohorts of patients. However, promising results have been obtained in some subgroups of patients that fail to respond to IFNα. Additionally, the good tolerability of IFNβ represents an important advantage of the drug. The rates of dropouts in controlled clinical trials, as well as the need for dose reductions or treatment discontinuation are very low. It might be worth assessing the value of IFNβ plus ribavirin in randomized studies with a larger cohort of patients, not eligible or not tolerating standard therapy, and for non-responders.

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Key words: Hepatitis C; Hepatitis C virus; Interferon beta; Ribavirin

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INTRODUCTION

In the absence of antiviral treatment, chronic hepatitis C (CHC) is a liver disease characterized by the development of necroinflammatory changes and progressive liver fibrosis leading to cirrhosis, end-stage liver disease and hepatocellular carcinoma. CHC represents a significant public health problem[1,2]. The objective of treatment is to eradicate infection and prevent progression to cirrhosis and associated complications of end-stage liver disease. According to the latest NIH Consensus Development Statement for the management of CHC, all patients with CHC are potential candidates for antiviral therapy[2]. The therapeutic strategy to be followed is currently well defined, mainly due to the significant progress that has occurred since the initial availability of Interferon-α (IFNα). The approval of ribavirin in combination therapy regimens with IFNα has dramatically improved therapy. Another advance was the introduction of pegylated IFNα, which allows a once-weekly subcutaneous administration and shows more favourable pharmacokinetics and greater efficacy. The highest sustained virological response (SVR) rates are attained with pegylated IFNα in combination with ribavirin[3]. Large randomised trials have shown efficacy of this combination in approximately half of the patients[4,5]. In spite of these advances, a number of patients do not respond to treatment with IFNα, or discontinue treatment due to the occurrence of adverse events associated with therapy.

IFNβ represents a potential therapeutic alternative for treatment of chronic viral hepatitis. In fact, in some countries, mainly in Japan, IFNβ already plays a role in therapeutic protocols. IFNβ belongs to the same family of glycoproteins as IFNα. Although both cytokines present different physicochemical and biological properties in many respects, IFNβ shares with IFNα some of the pharmacological characteristics thus indicated for treatment of chronic hepatitis C infection[6,7].

Three different types of IFNβ are available[8]: (1) Natural human IFNβ (nIFNβ) is produced by human fibroblasts, and is currently used in Japan for treating CHC; (2) Recombinant human IFNβ-1a (rhIFNβ-1a) is produced by mammalian cells and is identical to the IFNβ that occurs naturally in humans; (3) Recombinant human IFNβ-1b (IFNβ-1b) is produced by *E-coli* and has its cysteine at position 17 substituted by serine. RhIFNβ-1a has been reported to be less immunogenic and more potent than the other forms of IFNβ[9]. IFNβ can be administered

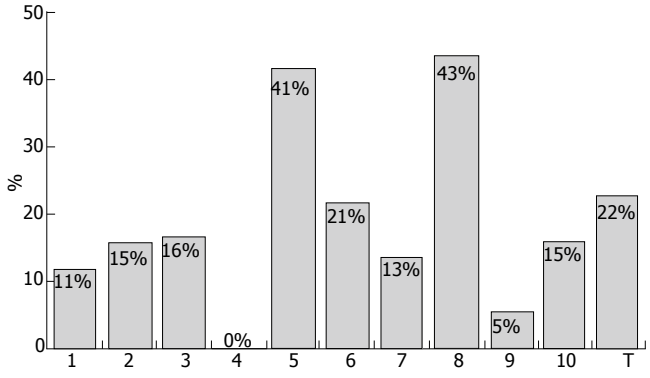


Figure 1 Sustained virological response rates in chronic hepatitis C naïve patients infected by genotype 1: studies with IFN-β administered intravenously. 1: Kainuma *et al*[16]. 2: Fukutomi *et al*[17]. 3: Kurosaki *et al*[18]. 4: Chemello *et al*[19]. 5: Kaito *et al*[20]. 6: Shiratori *et al*[21]. 7: Mochizuki *et al*[22]. 8: Kakizaki *et al*[23]. 9: Suzuki *et al*[24]. 10: Enomoto *et al*[25]. T: total [mean of studies].

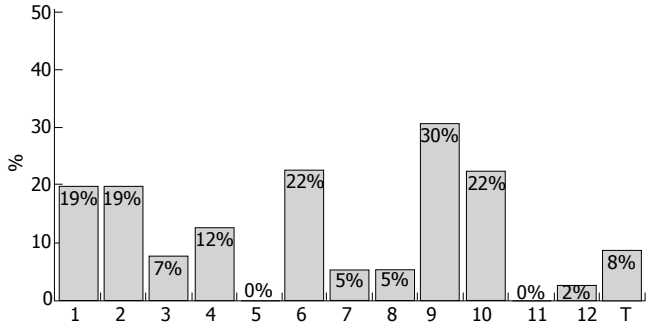


Figure 2 Sustained virological response rates in chronic hepatitis C genotype 1 naïve patients: studies with subcutaneous IFN-β.1: Habersetzer *et al*[26]. 2: Andrade *et al*[27]. 3: Castro *et al*[28]. 4: Castro *et al*[29]. 5: Villa *et al*[30]. 6: Kakumu *et al*[31]. 7: Perez *et al*[32]. 8: Bernardinello *et al*[33]. 9: Frosi *et al*[34]. 10: Pellicano *et al*[35]. 11: Nakamura *et al*[36]. 12: Fesce *et al*[12]. T: total [mean of studies].

intravenously (iv), intramuscularly (im) or subcutaneously (sc). Pharmacokinectic and pharmacodynamic studies[10] have shown that the extent and duration of the clinical and biologic effects of IFNβ are independent of the route of administration.

In this article we review the data currently available on the efficacy and safety of IFNβ for the treatment of CHC. A number of clinical trials have been conducted worldwide. However, many of them are pilot studies with small cohorts of patients targeting different subpopulations, thus frequently leading to controversial results. As a result, one has to be cautious when extracting conclusions. Distinct factors have been reported to influence efficacy of IFNβ in hepatitis C virus (HCV) infection, including the dose, frequency of administration, the dynamics of HCV clearance in the initial phases of treatment, patient age, pre-treatment viral load, HCV genotype and route of administration.

Several studies have been conducted to investigate the most appropriate doses and regimens of IFNβ for CHC showing, in general, that the highest doses did not have the greatest efficacy and result in poorer tolerability[11-14]. Generally, the intravenous administration achieved better SVR rates than subcutaneous route (Figures 1 and 2); furthermore, intravenous IFNβ obtains better results than recombinant IFNα monotherapy in CHC patients with genotype 1[12,15-36]. Similarly to other therapies with recombinant IFNα, patients infected with HCV genotype no-1 obtain higher SVR rates (Figures 1 and 3)[17,18,21-23,37]. In a randomized study with 92 CHC patients, a better biochemical response was observed in CHC patients treated with 6 million units (MIU) nIFNβ three times a week for 12 mo than in those administered 3 MIU (21% *vs* 4.5%, respectively)[12]. The frequency of administration also seems to play a role in the effects of IFNβ. Shiratori *et al* treated 22 CHC patients with high viral load and HCV subtype 1b with two different regimens of IFNβ: 6 MIU once a day, or 3 MIU twice a day[13]. The efficacy of the drug was higher with twice daily administration: negativity for HCV-RNA occurred in 18%, 73% and 89% of patients at 1, 2 and 3 wk, respectively, in the twice-a-day group, in contrast

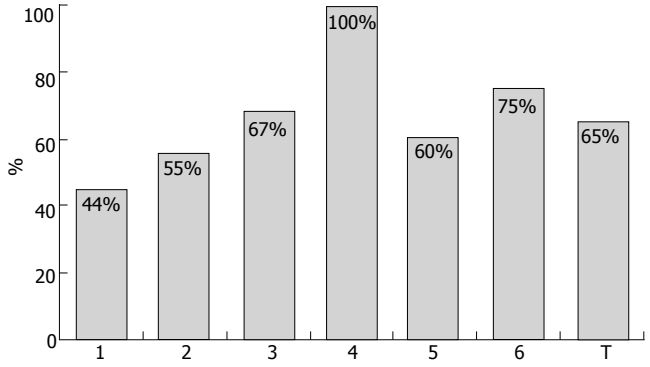


Figure 3 Sustained virological response rates in chronic hepatitis C naïve patients infected by genotype no-1: studies with intravenous IFN-β. 1: Fukutomi *et al*[17]. 2: Kurosaki *et al*[18]. 3: Shiratori *et al*[21]. 4: Nakamura *et al*[37]. 5: Mochizuki *et al*[22]. 6: Kakizaki *et al*[23]. T: total [mean of studies].

to 0%, 0% and 18% in the once-a-day group. However, the incidences of (a) reduced platelet counts and albumin levels; (b) increased serum ALT/AST; and (c) renal toxicity, were higher for the twice-a-day group. A similar outcome in terms of efficacy and safety was observed by Fujiwara *et al* in a randomized trial with 54 patients, treated either with 6 MIU every 24 or with 3 MIU every 12[14]: serum HCV-RNA disappeared in 95% of the patients on the twice-daily regimen, as compared with 74% in the once-daily injection group. Tolerability to IFNβ (in terms of proteinuria, thrombocytopenia and serum ALT levels elevation) was worse in patients receiving 3 MIU every 12 h. At the same time, in an other study, no differences between twice-daily and once-daily administrations were observed: Suzuki *et al* randomized 20 CHC patients with genotype 1b and high HCV-RNA level to receive either twice-daily 3 MIU IFNβ (group A) or once-daily 6 MIU IFNβ for 4 wk (group B)[24]. All patients then received a daily dose of 6 MIU IFNβ for 12 wk, followed by IFNα three times weekly for 16 wk; the treatment of group A was not superior to that of group B in terms of sustained responses, while adverse effects were more frequent and pronounced in group A.

The mechanisms underlying the possible enhanced an-

tiviral efficiency of frequent dosing of IFNβ were investigated in another study: serum HCV dynamics and double-stranded RNA-activated protein kinase (PKR) and MxA mRNA levels were monitored in peripheral blood mononuclear cells (PBMC) of 140 CHC patients randomized to receive twice daily 3 MIU IFNβ or once daily 6 MIU/d IFNβ[38]. The twice-a-day administration reduced the expression level of peak PKR and MxA gene expression in the first phase (4 h after single administration), although it was higher in the second phase. Furthermore, this regimen induced greater rates of HCV decline in the second phase. This enhanced clearance of HCV- infected cells probably represents the basis for the improved efficacy of frequent administration of IFNβ.

Fukutomi *et al* also studied the relation of HCV clearance during the initial phases of treatment with IFNβ with efficacy of treatment[39]. They showed that the decay slope calculated from HCV-RNA levels determined at 0 and 24 after the initiation of IFNβ treatment of CHC patients correlated with the proportion of sustained responders. Based on these findings, the authors proposed that the decay slope of viral clearance could be used as a predictor of the efficacy of therapy. However, further mechanistic data may be needed to confirm this possibility. Additional factors influencing sustained response to IFNβ have been identified[17]: in a study with 52 patients treated with 6 MIU/day IFNβ for 8 wk, the factors predicting efficacy of the treatment were primarily younger age and low pre-treatment viral load, and secondarily, HCV genotype 2a or 2b. The influence of low viral load in the outcome of therapy was further documented in a study in which 112 CHC patients received 6 MIU/d intravenous natural IFNβ for 12 wk[21]. In this trial, 88% of patients presenting a low pre-treatment viral load ($< 6.3\times10^5$ copies/mL), experienced a virological sustained response, as compared with only 22% patients that had a high viral load (in spite of the latter having the chance to receive an additional administration of IFNβ three times weekly for subsequent 14 wk at the patients' request).

Recently, Mochizuki *et al* have studied the effect of "*in vitro*" IFNβ on HCV in PBMC analysing whether this effect was associated with clinical response to IFNβ[22]. Twenty-seven patients with CHC were enrolled into this study. They were given intravenous administration of 6 MIU IFNβ daily for 6 wk followed by three times weekly for 20 wk. PBMC collected before IFNβ therapy were incubated with IFNβ and HCV-RNA in PMBC was semi-quantitatively determined. Eight patients (32%) had sustained loss of serum HCV-RNA with normal serum ALT levels after IFN therapy. Multiple logistic regression analysis revealed that the decrease of HCV-RNA amount in PBMC by IFNβ was the only independent predictor for complete response ($P<0.05$). The authors proposed that the effect of *in vitro* IFNβ on HCV in PBMC reflects clinical response and would be taken into account as a predictive marker of IFNβ therapy for CHC.

The route of IFNβ administration might also influence the outcome of treatment. Reports can be found in the literature describing intramuscular[12,29,30,32], subcutaneous[28,35,40] and intravenous injections[17,21,22,41-45]. Since there are no trials directly comparing the results of one route *vs* the other, no clear recommendations exist. The most appropriate route shoud be selected in each case to grant sufficient bioavailability, reduced toxicity, comfortable administration and adequate patient compliance.

Studies comparing the efficacy of IFNβ vs IFNα.

A number of studies have evaluated the performance of IFNβ *vs* IFNα in CHC patients[30,32,34,41,42,46,47]. Generally, IFNβ has led to poorer outcomes than IFNα, particularly when IFNβ was administered either through the intramuscular route[31,32] (probably due to low bioavailability or to insufficient dose levels), or with a once-daily regimen[46]. Only one small study reported a similar efficacy of IFNβ and IFNα when both were given subcutaneously, at a dose of 3 MIU, three times weekly for 6 mo[3]. However, the good tolerability of IFNβ in all studies suggests that further trials aimed at improving its clinical efficacy might produce interesting results. Asahina *et al* investigated the reason underlying the differences in clinical efficacy between both interferons by assessing HCV dynamics in serum and PBMC of patients treated with IFNα (alone or in combination with ribavirin) and IFNβ (twice-a-day or once-a-day administration)[31]. Although improved viral clearance dynamics were observed with combination or twice-daily dosing regimens (leading to increased rates of sustained eradication of HCV), no dramatic effects in the slopes of HCV decline or in HCV half-lives in the second phase were seen between IFNα and IFNβ.

IFNβ for patients not responding to IFNα

An interesting opportunity for IFNβ is its use as an alternative in patients not responding to IFNα. A randomized study with 200 clinically nonresponding CHC patients analysed the efficacy of intravenous IFNβ, in comparison with combination of IFNα and ribavirin for 12 wk[42]. The short-term treatment outcome showed a biochemical and virological response in 42% of patients treated with IFNβ *vs* 22% in patients treated with combination therapy. Sustained response (as documented after a further 48 wk) was seen in 21% and 13% of patients, respectively. These data indicate that, at least as a short-term therapy, IFNβ may offer a chance for sustained response in a subset of IFNα non-responders.

Cheng *et al* have evaluated the efficacy of IFNβ-1a in the treatment of CHC patients unresponsive to IFNα in a multicentre, randomized study[48]. A total of 267 patients were randomized to one of four groups: subcutaneous IFNβ-1a 12 MIU (44 μg) or 24 MIU (88 μg) administered 3 times weekly or daily. Patients were treated for 48 wk and then followed up for an additional 24 wk. There was a tendency towards a dose-response relationship regarding virological and biochemical response. Overall, 22 patients (8.3%) had a virological response at the end of treatment; 9 patients (3.4%) had a sustained virological response (SVR). Strikingly, 21.7% (5/23) of Chinese patients achieved SVR. Univariate analysis revealed that race was the only variable related to SVR (odds ratio 16.6; 35% CI 4.1 = 67.3; $P < 0.0001$). The authors concluded that IFNβ-1a

provided considerable clinical benefit in Chinese patients with CHC unresponsive to IFNα.

A few more pilot studies (with cohorts of between 10 and 30 patients) have been conducted to evaluate the efficacy of IFNβ in CHC non-responder patients[40,43,44,49]. Although these have confirmed that some patients may benefit from IFNβ therapy in terms of early biochemical and viral responses (between 20% and 40%, depending on the doses and regimens), sustained responses have been very seldom observed. None of the studies has shown a clear advantage for patients infected with any particular HCV genotype. Pellicano *et al*[40] treated 30 non-responders with 12 MIU subcutaneous IFNβ, three times weekly for 3 mo. Patients responding were given 12 MIU IFNβ for a further 3 mo, and those showing no biochemical and viral improvement had their dose increased to 18 MIU for the same 3 mo. While 20% of patients exhibited a response at the end of treatment, only one patient presented a sustained virological response at the end of post-treatment follow-up. In relapser patients after IFNβ monotherpay, few studies exist. Nomura *et al* treated 43 relapser patients genotype 1 CHC after IFNβ monotherapy, with 6 MIU of IFNβ, achieving a sustained virological response of 21%. Authors also found that better results were obtained when therpay with IFNβ was given near relpase[50]. In summary, trials with IFNβ monotherapy for CHC patients resistant to IFNα have shown conflicting results. Tolerability was good in most cases, but further investigation on strategies to improve efficacy of the drug are clearly needed.

Combination therapy: IFNβ plus ribavirin

There is limited information about the efficacy of IFNβ in combination with ribavirin for the treatment of CHC patients. In a pilot study, 27 patients were randomized to receive either 800-1000 mg/d ribavirin or IFNβ alone (3 MIU, three times weekly), or combination of the two for 24 wk[31]. No significant differences in terms of sustained viral response were observed between patients treated with IFNβ monotherapy or in combination with ribavirin (2 of 9 patients, respectively). Tolerability was good in all groups. The limited number of patients included in the study hampers reaching conclusions about the benefits of combination therapy. Pellicano *et al*[41] randomized 102 naïve CHC patients to receive 6 MIU of recombinant human IFNβ-1a subcutaneously every day for 24 wk alone or in combination with ribavirin (1000 mg/d in patients < 70 kg and 1200 mg/d in patients > 70 kg).The end-of-treatment virological response rate was 29.4% for monotherapy and 41.2% for combination therapy. After an additional 24 wk follow-up period, the sustained virological response rate in both groups was 21.56% and 27.45%, respectively. Tolerability was good, without major differences between groups (except for 2 patients in the combination therapy group who had to stop therapy due to drug-related adverse events). These results suggest an improvement of efficacy by combination therapy with respect to monotherapy. Other associations with IFNβ (amantadine, adelavin-9) do not obtain better results than those reached with ribavirin[36,51,52].

Clinical trials of IFNβ in special subpopulations of patients

Acute hepatitis C. Several studies and case reports dealing with the efficacy and safety of IFNβ in patients with acute hepatitis C have been published[45,53,54]. Omata *et al* randomized 25 acute non-A, non-B hepatitis patients to receive or not an average of 52 MIU IFNβ for 30 d, and in those showing elevated serum aminotransferase concentrations after one year, a second course of IFNβ was administrated[53]. Ten out of 11 treated patients showed normalized serum aminotransferase and negative HCV RNA at 3-years follow-up, as compared with 3 out of 14 untreated patients. Tadano and cols. Treated 97 non-A, non-B hepatitis cases with different regimens of IFNβ, reporting a resolution rate of 32% among patients with positive HCV RNA[54]. The dose of 336 MIU produced the highest resolution rate (83%; 10/12 of patients treated with this schedule). Lower doses of IFNβ induced resolution rates of 0% to 38%. These data suggest that IFNβ may prevent the progression of acute non-A, non-B hepatitis to chronically by eradicating HCV.

Cirrhosis. According to the currently available data, IFNβ is not efficacious for the treatment of patients with HCV-related cirrhosis. Sixty-one patients were randomized to receive or not 6 MIU IFNβ twice-a-week for 6 mo followed by 3 MIU/tiw for 6 mo, and followed-up for 5 years[33]. End-of-treatment biochemical and virological responses were observed in 13% and 11% of treated patients. At long-term follow-up, 16% of treated and 17% of untreated patients presented normalized serum alanine aminotransferase levels, whereas only 5% and 4% respectively, presented a virological response. Additionally, no significant reduction of cirrhosis-related clinical events (variceal bleeding, ascites, hepatic encephalopathy, progression to hepatocellular carcinoma), was associated with treatment.

IFNβ for other chronic liver diseases. IFNβ might also represent an appropriate therapy for chronic hepatitis B patients not responding to IFNα. In a pilot study, Muñoz *et al* treated 29 such patients with 6 MIU IFNβ, five times a week for 24 wk, and followed-up the patients for 48 wk[55]. End-of-treatment biochemical and virological response was obtained in 38% of patients and sustained virological response occurred in 21% of patients. The therapy was well tolerated and safe. The results were particularly positive for patients with HbeAg-negative/HBV DNA-positive chronic hepatitis B.

Safety of IFNβ

As already mentioned throughout this review, IFNβ generally presents a good tolerability, even in combination with ribavirin. This constitutes one of the main strengths of this drug as an alternative to other CHC therapies. The fact that IFNβ is the treatment of choice for relapsing-remitting multiple sclerosis, a chronic disease requiring high doses and frequent administration for years, has resulted in the availability of abundant information on this drug's safety. Severe side effects are unfrequent and several clinical studies in CHC patients report no requirement for treatment discontinuation and/or dose modifications. The

adverse effects most frequently encountered during IFNβ therapy are flu-like syndrome, fever, fatigue and injection-site reactions. The drug seems to be equally well tolerated by responders and non-responders. The frequency of adverse events is also similar among subpopulations such as patients with genotype-1b HCV hepatitis unresponsive to IFNα treatment or with HCV-related cirrhosis and patients with acute viral hepatitis. The interested reader is referred to a recently published review updating the available information about safety of IFNβ treatment for CHC[56].

SUMMARY

HCV infection remains an important health problem. Although the therapeutic options have experienced dramatic improvements during recent years, treatment of the disease is still far from optimal, particularly for those subpopulations that do not respond to the standard combination therapy with IFNα and ribavirin. Although in some cases the use of higher doses or longer treatment periods may be effective, these approaches are generally associated with a higher incidence of adverse effects, which may either lead to a reduction in patient compliance or require drug withdrawal. IFNβ could represent an interesting alternative for treating CHC in these patients. A review of the literature shows controversial data about IFNβ efficacy in CHC, the main reason being that many results stem from pilot studies with small cohorts of patients. However, promising results have been obtained in some subgroups of patients that fail to respond to IFNα. Additionally, the good tolerability of IFNβ represents an important advantage of the drug. The rates of dropouts in controlled clinical trials, as well as the need for dose reductions or treatment discontinuation are very low. All these data suggest that it might be worth assessing the value of IFNβ in randomized studies with larger cohorts patients, with special emphasis on the combination with ribavirin. IFNβ may represent an efficacious and safe second-line therapy for those populations of patients not eligible or not tolerating standard therapy, as well as for non-responders.

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BASIC RESEARCH

Splenic vasculopathy in portal hypertension patients

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Abstract

AIM: To investigate the interaction between portal hypertension, splanchnic hyperdynamic circulation and splanchnic vasculopathy by observing splenic arterial and venous pathological changes and the ro1e of extracellular matrix in the pathogenesis of portal hypertensive vasculopathy by measuring the expression of type I and type III procollagen mRNA in splenic venous walls of portal hypertensive patients.

METHODS: Morphological changes of splenic arteries and veins taken from portal hypertensive patients ($n = 20$) and normal controls ($n = 10$) were observed under optical and electron microscope. Total RNA was extracted and the expression of type I and type III procollagen mRNA in splenic venous walls of portal hypertensive patients ($n = 20$) was semi-quantitatively detected using reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Under optical microscope, splenic arterial intima was destroyed and internal elastic membrane and medial elastic fibers of the splenic arterial walls were degenerated and broken. Splenic venous intima became remarkably thick. Endothelia1 cells were not intact with formation of mural thrombus. The tunica media became thickened significantly due to hypertrophy of smooth muscles. Fibers and connective tissues were increased obviously. Under electron microscope, smooth muscle cells of the splenic arteries were degenerated and necrotized. Phenotypes of smooth muscle cells changed from constrictive into synthetic type. Red blood cells and platelets accumulated around the damaged endothelial cells. Synthetic smooth muscle cells were predominant in splenic veins and their cytoplasma had plentiful rough endoplasmic reticulum ribosomes and Golgi bodies. Along the vascular wall, a lot of collagen fibers were deposited, the intima was damaged and blood components accumulated. There was no significant difference in the expression of type I procollagen mRNA in splenic venous wall between the patients with portal hypertension and those without portal hypertension ($P > 0.05$), but the expression of type III procoagen mRNA was significantly stronger in the patients with portal hypertension than in those without portal hypertension ($P < 0.01$).

CONCLUSION: Type III procollagen and collagen might be important extra-cellular matrix resulting in neointimal formation and vascular remodeling in the pathogenesis of portal hypertensive vasculopathy. The pathological changes in splenic arteries and veins exist in portal hypertension patients. There might be an interaction between portal hypertension, splanchnic hyperdynamic circulation and splanchnic vasculopathy.

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Key words: Portal hypertension; Splanchnic hyperdynamic circulation; Splanchnic vasculopathy

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INTRODUCTION

With the elevation of portal pressure, obvious pathological changes in splanchnic vessels occur, such as extensive formation of porto-systemic communicating branches, remodeling of splanchnic great arteries and veins, which are defined as portal hypertensive vasculopathy (PHV). PHV is compensation of splanchnic vessels for hyperdynamic circulation, but also aggravates the portal pressure. PHV is one of the direct causes of variceal hemorrhage[1,2]. In this study the splanchnic vessels in portal hypertensive patients were observed under optical and electron microscope in order to explore the interaction between portal hypertension, splanchnic hyperdynamic circulation and splanchnic vasculopathy. The expression of types I and III procollagen mRNA in the splenic venous wall was detected by RT-PCR in the patients with portal hypertension in order to explore their ro1e in the pathogenesis of portal hypertensive vasculopathy.

MATERIALS AND METHODS

Patients

From June 2002 to October 2002, 20 patients including 15 males and 5 females with a mean age of 41 years with portal hypertension were selected from Center of Hepatic Surgery of Tongji Hospital and Caidian Schistosomiasis Prophylactic therapeutic Institution. Among them 14 belonged to Child class A and 6 to Child class B. Endoscopic examination revealed that all patients had moderate or serious gastroesophageal varices accompanied with hypersplenism and splenomegaly. Pathologically, there were 12 cases of schistosomial hepatic cirrhosis and 8 cases of post-hepatitis hepatic cirrhosis. Eight cases had a history of one or more episodes of massive upper digestive tract hemorrhage. Eight cases received splenectomy in combination with esophagogastric devascularization and twelve cases splenectomy. The control group I included 10 patients (6 males and 4 females with a mean age of 38 years) with traumatic spleen rupture without liver cirrhosis and all were subjected to emergency splenectomy. A piece of splenic arteries and veins near the hilus of the spleen was removed during the operation under laser scanning confocal microscope (Germany). PCR device (PTC-200 type) was the product of MJ Research Inc., (USA). UVP gel imaging analysis system was the product of UVP Company (England). M-MLV reverse transcriptionase, Oligo(dT)15 primer, DNTP, TaqNa polymerase were the products of Promega Company(USA). PCR marker was the product of Dalianbao Bioengineering Company.

Observation under optical and electron microscope

The vascular specimens were fixed with 40 g/L neutrally buffered formaldehyde, routinely embedded with paraffin, cut into 5μm thick sections and stained with H&E. The sections were observed under microscope. The fresh vascular specimens were cut into 1mm x 1mm x 1mm senctions, put into 25 mg/L glutaraldehyde within 1 min for pre-fixation for 2h, washed with phosphate buffer (pH 7.4), post-fixed with 10g/L osmic acid at 4℃ for 2h, then dehydrated with ascending concentrations of alcohol and acetone, embedded with epoxy resin Epon812, and finally cut into ultrathin sections. The sections were double stained with plumbum and observed under a transmission electron microscope (Opton EM l0C Model). After dehydration with alcohol, the sections were dried at a critical point, sherardized in vacuum, then examined and photographed under a Nikon scanning electron microscope(S-520 model).

Expression of type I and III procollagen mRNA

RT-PCR primers were designed according to Primer 5.0 version [human (a1) type I proco1 1agen (GenbankZ-74615): upstream primer 5' -ACCGCAAACCTTTCTACTTC-3' and downstream primer 5'-AAGGCGAGTAGGAGCAGT-3', 465 bp; human (al) type III procollagen (GenbankX-14420): upstream primer 5'-ACCGCAAACCTTTCTACTTC-3' and downstream primer 5'AAGGCGAGTAGGAGCAGT-3', 384 bp; human GAPDH (Genbankm 33197) served as house-keeping gene internal reference; upstream primer 5'-GGATTTGGTCGTATTGGG-3'and downstream primer 5'-GGAAGAGGTGAGGGATT-3', 384 bp]. The total RNA from splenic veins was extracted by Trizol one-step method. In each sample containing 5 mg RNA, DEPC was added to a volume of 27 μL following addition of 15 μL (1 ug) o1igo (dT). After degeneration of RNA by incubation at 70℃ for 5 min, 5×8 μL buffer, 4×2 μL dNTP, 1 μL RNAase inhibitor, 2 μL MMLV (400U) were added. In a total vo1ume of 40 μL, reverse transcription was performed at 42℃ for 1h fo11owed by denaturation at 95℃. A total volume of PCR mixture contained 10×5 μL Buffer, 3 μL 25 mmo1 $MgCl_2$, 4 x 1 μL dNTP (10pmol/mL), 5 μL upstream (10pmo1/L), 5 μL cDNA, 1 μL Taq enzyme (3 U), 33 μL DEPC. PCR conditions were 30 cycles of annealing at 52℃ for human type I and III pro collagen, 30 cycles of annealing at 51℃ for GAPDH. The PCR amplified products (10mL) were spotted and electrophoresed on 15g/L gel at 5V/cm. The results were observed under ultra-violet light. UVP gel imaging analysis system (England) was used to analyze the density of electrophoresis bands of PCR products. The targeting gene expression intensity = targeting gene RT-PCR product A÷GAPDH RT-PCR product A

Statistical analysis

All data were expressed as mean ± SD. Homogeneity test of variance, ANOVA and t test were performed by SPSSll.0 statistical software. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology

As compared with the patients without hepatic cirrhosis, spleen of the patients with hepatic cirrhosis was significantly enlarged, splenic arterial and venous walls were obviously thickened, vascular caliber was increased and elasticity was decreased. Under optical microscope, intima of the normal splenic arterial walls was smooth, endothelial cells were intact and no subintimal smooth muscle cells were seen. In the tunica media, multiple layers of smooth muscle cells were separated by collagenous and elastic fibers. In the patients with hepatic cirrhosis, intima of the splenic artery was significantly thickened, endothelial cells were not intact, internal elastic membrane was broken and smooth muscle cells in tunica media were disturbed (Figures 1A and 1B). Intima of the normal splenic veins was intact and endothelial cells were not destroyed. In the patients with hepatic cirrhosis, intima of the splenic vein was locally thickeried, endothelial cells were not intact, intima was damaged and mural thrombus formed (Figures 1C and 1D). Under electron transmission microscope, smooth muscle cells of the normal splenic artery were fusiform and nuclei were long-circular in shape. In cytoplasma, there were myofilaments, mitochondria, endoplasmic reticulum ribosomes, Golgi bodies and gluycogenic granula. In the patients with hepatic cirrhosis, endothelial tissue of the splenic artery was damaged, internal elastic membrane was broken and elastic fibers were ruptured. In the tunica media, smooth muscle cells increased and migrated into subintima. Partial smooth muscle cells had atrophy and degeneration. Local

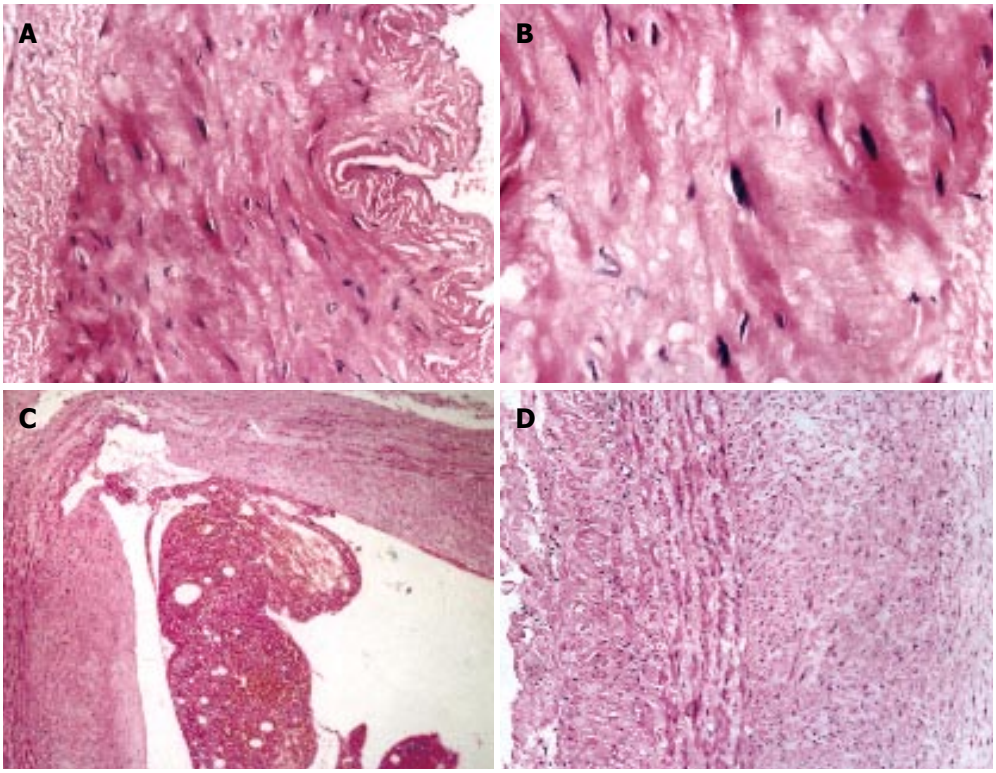


Figure 1 Changes of splenic arteries (**A**, **B**) and splenic veins (**C**, **D**) in patients with portal hypertension.

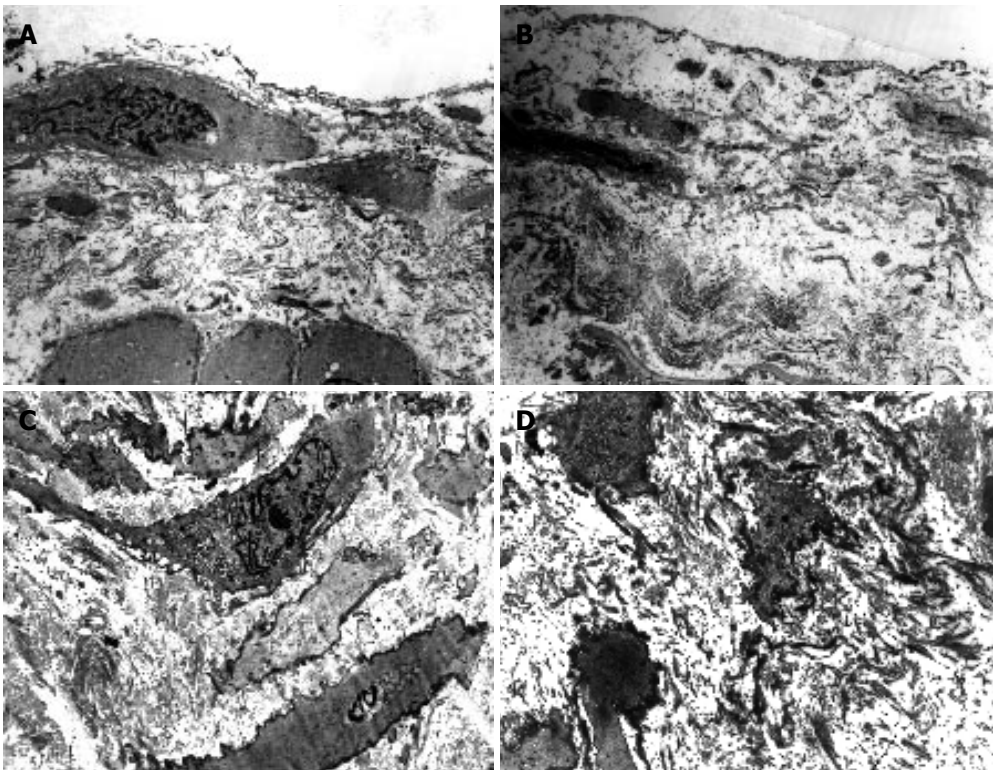


Figure 2 Ultrastructure of splenic artery (**A**, **B**) and splenic vein (**C**, **D**) in patients with portal hypertension.

cytoplasma was necrotized and formed vacuoles. The number of mitochondria was increased, their cristae disappeared and vacuoles formed. Collagenous fibers along the vascular wall were obviously increased and arranged disorderly. In some smooth muscle cells, rough endoplasmic reticulum, ribosomes and Golgi bodies were increased while myofilaments reduced, indicating that phenotypes of smooth muscle cells were changed from constrictive to synthetic type (Figures 2A and 2B). Intima of the normal splenic vein was smooth and endothelial cells were not damaged. In the patients with hepatic cirrhosis, intima of the splenic vein wall was damaged and vascular smooth muscle cells of synthetic type were predominant. Cytoplasma of the smooth muscle cells had plentiful rough endoplasmic reticulum and Golgi bodies. Along the vascular wall, a lot of collagenous fibers were deposited and disorderly arranged (Figures 2C and 2D). Under scanning electron microscope, intima was smooth

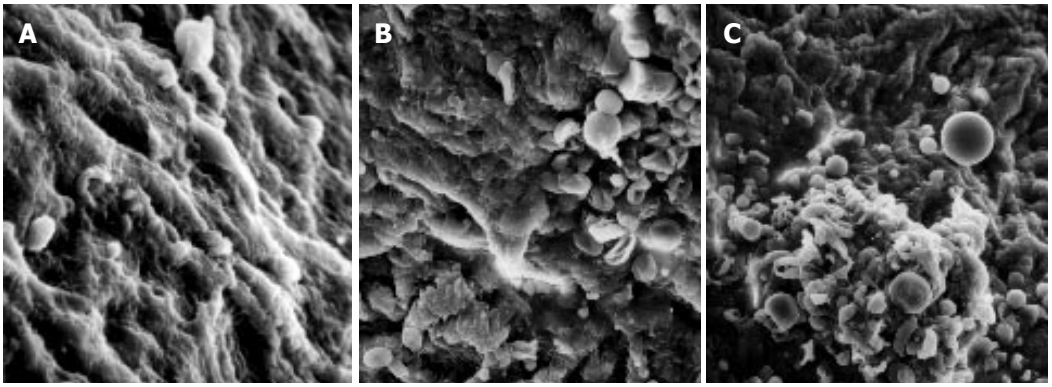


Figure 3 Observation of splenic vein in patients with traumatic splenic rupture without liver cirrhosis (**A**), in patients with portal hypertension (**B**), and in patients with portal hypertension (**C**) under scanning electron microscope.

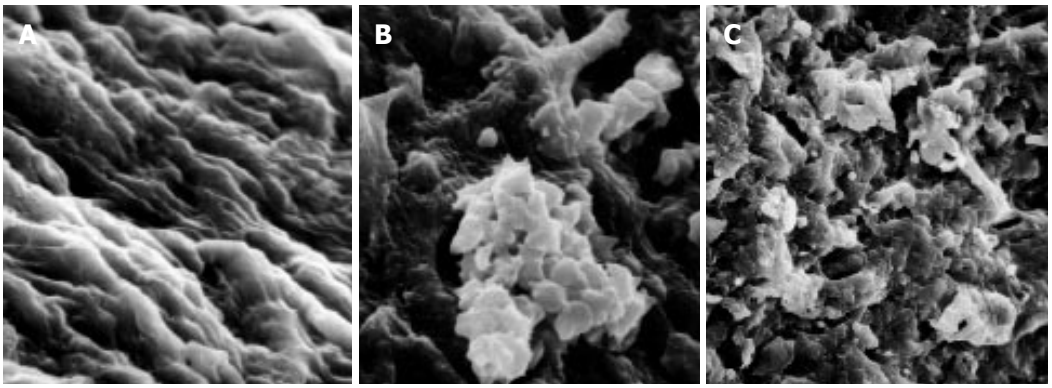


Figure 4 Observation of splenic artery in patients with traumatic splenic rupture without liver cirrhosis (**A**), in patients with portal hypertension (**B**), and in patients with portal hypertension (**C**) under scanning electron microscope.

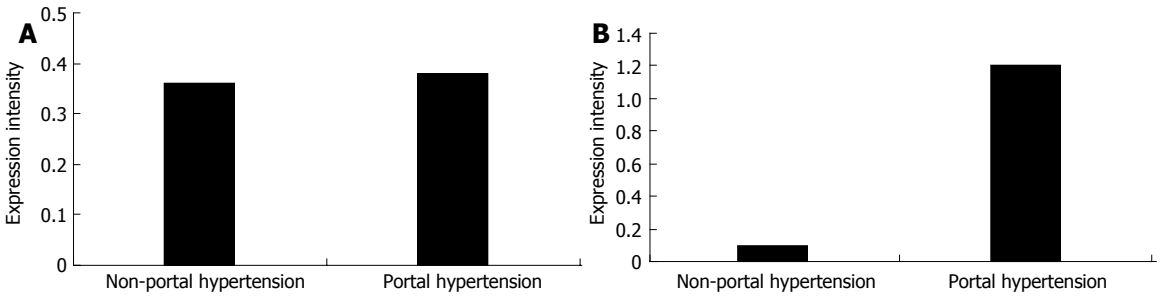


Figure 5 Comparison of the expression intensity of type I (**A**) and type III (**B**) procollagen mRNA in splenic vein wall of patients with and without portal hypertension.

and endothelial cells were intact in the normal splenic vein (Figure 3A). In the patients with hepatic cirrhosis, intima of the splenic vein was damaged, endothelial cells were not intact, blood components accumulated, surface of the endothelia was irregular(Figures 3B and 3C). In the normal splenic artery, intima was intact and endothelial cells were not destroyed (Figure 4A). In the patients with hepatic cirrhosis, intima of the splenic artery was damaged and red blood cells and platelets accumulated around the damaged area (Figures 4B and 4C).

Expression of type I and III procollagen mRNA

In splenic vein wall of the patients with portal hypertension, the expression intensity of type I procollagen mRNA was 0.3868 ± 0.0358, while that in the patients without hepatic cirrhosis was 0.3642 ± 0.0332 with no significant difference ($P > 0.05$). In portal vascular wall of the patients with portal hypertension, the expression intensity of type III procollagen mRNA was 1.1855 ± 0.2140, while that in the patients without hepatic cirrhosis was 0.1095 ± 0.1558 with significant difference ($P < 0.01$, Figures 5, 6)

DISCUSSION

Serious damage to the intima in patient with portal hypertension results from increased portal pressure and blood flow. It has been reported that phenotype of vascular smooth muscle cells changes from normal constrictive to synthetic type[3,4] due to increased cytokines[5,6], growth factor[7], shearing force[8,9], oxygen stress[10]. The expression of constrictive proteins in vascular smooth muscle cells is inhibited, and a plentiful of extra-cellular matrix (ECM) is produced and migrates into subintima. In this study, under optical microscope, the tunica media was thickened, which is an adaptative compensatoty response of vessels to the increased pressure. Smooth muscle cells in tunica media of the splenic artery were degenerated and became shrunk and their phenotype changed from constrictive to synthetic type, which is attributed to the ischemic vascular wall injury by insufficient blood supply due to compression of nutrient vessels resulting from the increased pressure

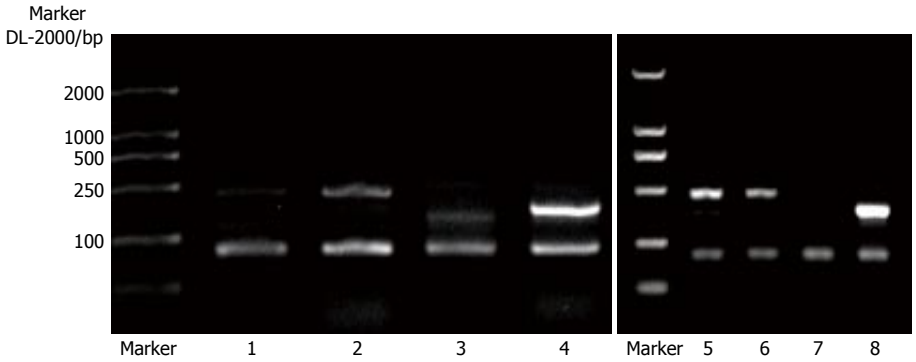


Figure 6 Expression of type III procollagen mRNA of splenic vein wall in patients with portal hypertension. Bands 1, 3, 5 and 7: control samples from patients without portal hypertension; Bands 2, 4, 6 and 8: samples from patients with portal hypertension.

and flow. Under scanning electron microscope, damage to intima of the splenic artery was seen, which is associated with the portal system involvement. In addition, under optical microscope, internal elastic membrane of the splenic artery was broken due to the following causes, such as splanchnic hyperdynamic circulation, increased blood flow and velocity, increased wall pressure and shear stress on vascular wall, long-term vascular congestion and dilation, thus resulting in endothelial exfoliation and rupture of elastic membrane. The broken internal elastic membrane contributes to the decreased ability of vascular wall to resist the blood flow attack. When the blood flow is increased, the vessels are not constricted and the blood flow is not regulated by elastic membrane, which is beneficial to the development of hyperdynamic circulation.

Collagen is an important vascular extra-cellular matrix and can maintain the flexibility and intensity of vascular wall. In this study, expression of type III procollagen mRNA in the splenic vein wall was about 5-6 times higher in patients with portal hypertension than in those without portal hypertension ($P<0.01$), while there was no significant difference in the expression of type I procollagen mRNA between the patients with and with portal hypertension ($P>0.05$). We believe that the high expression of type III porcollagen mRNA in patients with portal hypertension might be an important factor promoting vascular remodeling. At present, it is unclear about the modulation mechanism of type I and III procollagen gene expression. It has been reported that many factors, such as FGF[11], Ang-II[12] can up-regulate type I and III procollagen mRNA in vascular smooth muscle cells.

In conclusion, type III porcellagen and collagen may be an important extracellular matrix resulting in vascular remodeling in patients with portal hypertension. Splenic vasculopathy exists in patients with portal hypertension, while intrahepatic blood flow obstruction resulting from hepatic cirrhosis is the initial factor responsible for the development of portal hypertension. On the one hand, portal hypertension can promote the formation and development of splanchnic hyperdynamic circulation and splanchnic vasculapathy. On the other hand, splanchnic vasculapathy can enhance the formation and development of portal hypertension, as well as promote splanchnic hyperdynamic circulation. These three factors play an important role in the pathogenesis of cirrhotic portal hypertension and are involved in variceal hemorrhage.

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BASIC RESEARCH

Pharmacokinetics and bioequivalence of ranitidine and bismuth derived from two compound preparations

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Abstract

AIM: To evaluate the bioequivalence of ranitidine and bismuth derived from two compound preparations.

METHODS: The bioavailability was measured in 20 healthy male Chinese volunteers following a single oral dose (equivalent to 200 mg of ranitidine and 220 mg of bismuth) of the test or reference products in the fasting state. Then blood samples were collected for 24 h. Plasma concentrations of ranitidine and bismuth were analyzed by high-performance liquid chromatography and inductively coupled plasma-mass spectrometry (ICP-MS), respectively. The non-compartmental method was used for pharmacokinetic analysis. Log-transformed C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ were tested for bioequivalence using ANOVA and Schuirmann two-one sided *t*-test. T_{max} was analyzed by Wilcoxon's test.

RESULTS: Various pharmacokinetic parameters of ranitidine derived from the two compound preparations, including C_{max}, $AUC_{(0-t)}$, $AUC_{(0-\infty)}$, T_{max} and $T_{1/2}$, were nearly consistent with previous observations. These parameters derived from test and reference drug were as follows: C_{max} (0.67 ± 0.21 *vs* 0.68 ± 0.22 mg/L), $AUC_{(0-t)}$ (3.1 ± 0.6 *vs* 3.0 ± 0.7 mg/L per hour), $AUC_{(0-\infty)}$ (3.3 ± 0.6 *vs* 3.2 ± 0.8 mg/L per hour), T_{max} (2.3 ± 0.9 *vs* 2.1 ± 0.9 h) and $T_{1/2}$ (2.8 ± 0.3 *vs* 3.1 ± 0.4 h). In addition, double-peak absorption profiles of ranitidine were found in some Chinese volunteers. For bismuth, those parameters derived from test and reference drug were as follows: C_{max} (11.80 ± 7.36 *vs* 11.40 ± 6.55 μg/L), $AUC_{(0-t)}$ (46.65 ± 16.97 *vs* 47.03 ± 21.49 μg/L per hour), T_{max} (0.50 ± 0.20 *vs* 0.50 ± 0.20 h) and $T_{1/2}$ (10.2 ± 2.3 *vs* 13.0 ± 6.9 h). Ninety percent of confidence intervals for the test/reference ratio of C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ derived from both ranitidine and bismuth were found within the bioequivalence acceptable range of 80%-125%. No significant difference was found in T_{max} derived from both ranitidine and bismuth.

CONCLUSION: The two compound preparations are bioequivalent and may be prescribed interchangeably.

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Key words: Ranitidine; Bismuth; Compound preparation; Bioequivalence; Pharmacokinetics; Healthy volunteers; High-performanace liquid chromatography; Inductively coupled plasma-mass spectrometry

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INTRODUCTION

Ranitidine is a very common histamine H_2 receptor antagonist. It exerts most prominent effect on basal acid secretion and less profound effect on acid production[1,2]. Bismuth potassium citrate has mucosal protective effects by blocking pepsin activity, retarding hydrogen-ion back-diffusion and stimulating prostaglandin synthesis. In addition, it has an inhibitory effect on *Helicobacter pylori* (*H pylori*)[3-6]. Thus, ranitidine and bismuth potassium citrate have a synergistic effect and are usually concurrently prescribed in poly-therapy regimens for gastroduodenal ulcers and *H pylori* infection. Especially, the availability of ranitidine bismuth citrate and compound preparation of ranitidine and bismuth potassium citrate in market makes administration more conveniently and increases medication compliance in ulcer patients[7,8].

The pharmacokinetics of ranitidine has been described previously[9-11]. After oral administration 50% of ranitidine is absorbed, resulting in the peak serum concentration of 2 ± 3 h after dosing, followed by elimination with a half-life of 2.5 ± 3 h. Ranitidine is metabolized to a small extent, primarily via flavin mono-oxygenases[12]. It is eliminated through kidney, with approximately 70% of the systemically available dose recovered in urine as unchanged drug. Bismuth is only minimally absorbed and its bioavailability ranges 0.16%-0.28%, but the median peak bismuth concentration occurs 30 min (range 15-105

min) post-dosing[13,14]. The concentration of bismuth in kidneys is high and it is also retained there for a long time. Elimination from blood displays multi-compartment pharmacokinetics[15].

Comparative bioavailability of different mono-preparations of ranitidine hydrochloride or colloidal bismuth sub-citrate in healthy volunteers or patients has been described previously[16-20]. However, to our knowledge, there is no report on the pharmacokinetic and bioequivalence evaluation of compound preparations containing these two drugs.

MATERIALS AND METHODS

Drugs and reagents

Ranitidine reference substance (purity> 99.5%) was provided by Shuguang Pharmaceutical Factory (Beijing, China). Bi_2O_3 reference substance (purity> 99.999%) and TlCl reference substance (purity> 99%) were provided by ALDRICH Chemical Company (USA). Nitric acid was of MOS grade. Ultra pure water was obtained from Milli-Q Academic (Millipore Co., USA). Acetonitrile was of HPLC grade. Potassium dihydrogen phosphate and perchloric acid were of analytical grade. Test preparation was compound ranitidine tablet (lot 041201, expiracy: 12/2006) from Chongqing Pharmaceutical Factory (Chongqing, China). Reference preparation was compound ranitidine capsule (lot 041002, expiracy: 9/2006) from Nuode Pharmaceutical Factory (Jiangsu, China). Each tablet or capsule contained ranitidine hydrochloride (equivalent to 100 mg of ranitidine) and bismuth potassium citrate (equivalent to 110 mg of bismuth).

Study subjects

After approval by the Ethics Committee of the 2nd Affiliated Hospital (School of Medicine, Zhejiang University), 20 male healthy Chinese volunteers (age, 24±1 years; weight, 63.8±6.1 kg; BMI, 22.1±1.7 kg/m^2) gave their written informed consent to participate in the study and all of them completed the study. All volunteers were considered to be in good health on the basis of physical examination, electrocardiogram (ECG), and laboratory tests including complete blood count, blood biochemistry testing and urinalysis. Each volunteer was required to be 18-40 years old, a nonsmoker, and to have a BMI between 19 and 25 kg/m^2. Participants were excluded for the following reasons: any significant medical history, history of any localized or systemic infection within 4 wk before admission, use of prescription or over-the counter medication or alcohol within 2 wk before enrollment, history of alcohol or drug abuse, donation of blood within the past 2 mo.

Study design

The study had an open-label, randomized, two-period crossover design, with 1 wk washout between periods. All volunteers were not allowed to take any medications 2 wk before and during the study period. After fasting for l2 h overnight, the volunteers received a single oral dose (equivalent to 200 mg of ranitidine and 220 mg of bismuth) of the test or reference products with 200 mL water in a crossover fashion. No food was allowed until 4 h after dose administration. Water intake was allowed after 2 h of dose. Water, lunch and dinner were given to all volunteers according to a time schedule. The volunteers were under direct medical supervision at the study site. Blood samples (4 mL) were drawn into Vacutainer™ tubes containing K_2EDTA from a forearm vein using an indwelling catheter before drug intake and at 0.17, 0.33, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 24 h for the two phases after dosing. Blood samples were centrifuged at 3000 r/min for 10 min, and plasma was separated and stored at -80 ℃ until assay. ECG, biochemical and hematological laboratory tests were performed for each volunteer to record any clinically abnormal finding.

Analytical procedure

High performance liquid chromatography (HPLC) assay method for ranitidine was developed in our laboratory. In brief, 50 μL of perchloric acid solution (2.5 mol/L) was added to 200 μL plasma in the eppendorf tube (1.5 mL). The mixture was vortexed for 20 s and then centrifuged at 36 670 r/min for 10 min. The supernatant was transferred to vial on the rack of autosampler and 50 μL was injected onto the column. The HPLC injection sequence was as follows: calibration standards of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 mg/L, volunteers' plasma samples and quality-control samples of 0.04, 0.4 and 1.5 mg/L throughout all sequences.

The assay method for bismuth was as follows: plasma samples (0.5 mL) were added to Tl (internal standard) working solution and diluted to a volume of 5 mL with super pure water, followed by direct measurement in a PQ3 ICP-MS system (Thermo Electron Corporation, USA). The assay sequence was as follows: calibration standards of 0.2, 0.5, 1.0, 5.0, 10.0, 30.0, 60.0, 100.0 μg/L, volunteers' plasma samples and quality-control samples of 0.30, 8.00, 20.0, 80.00 μg/L throughout all sequences.

The two assay methods were validated according to international guidelines[21].

Chromatography

Chromatography for ranitidine was performed on a Beckman SYSTEM GOLD® liquid chromatography system (USA) consisting of a 125 solvent module, 508 auto-sampler, 166 UV detector and AT-330 column oven (Medilab). The software package 32 Karat 5.0 was used to control the chromatographic system and produce UV-spectrometric data. Analytical column used was Kromasil C18 ODS (Sweden, 250×4.6 mm ID, 5 μm, particle size), protected by a security guard cartridge C18 ODS 4×3.0 mm ID (Phenomenex Inc, USA). The mobile phase consisted of 0.02 mol/L KH_2PO_4-acetonitrile (86:14, V/V). The flow rate was 1.0 mL/min. Column oven was kept at 25 ℃. Auto-sampler was set at 4 ℃. The UV detective wavelength was 320 nm.

Working condition of ICP-MS

ICP-MS measurements were carried out under the following operation conditions. The forward power was 1350 W, the coolant and auxiliary and nebulizer gas

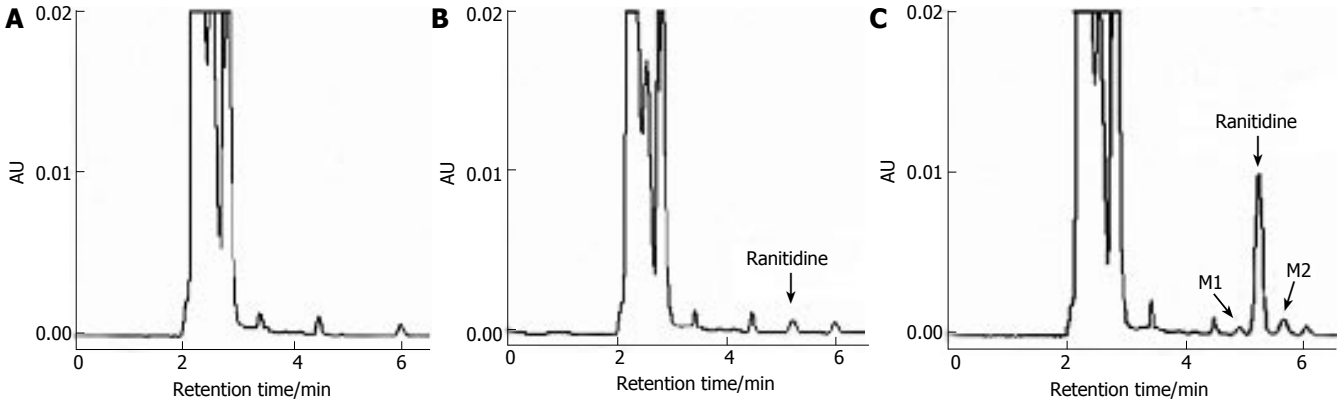


Figure 1 Chromatograms of spiked plasma sample (**B**), plasma samples of patients before (**A**) and after (**C**) the administration of 200 mg ranitidine. M1 and M2 are metabolites of ranitidine.

flow rate were 13, 0.70, and 0.73 L/min, respectively. Sample uptake flow rate was 1.2 mL/min. Mass-to-charge ratios detected (m/z) were 205 (^{205}Tl) and 209 (^{209}Bi). Sweep mode was peak hopping. Dwell time was 10 ms. Number of sweeps per run was 60. Uptake time was 60 s. Acquisition duration was 4 s.

Statistical analysis

Pharmacokinetic parameters were calculated by non-compartmental method. Bioequivalence evaluation was performed by the software BAPP2.0 (Center of Drug Metabolism & Pharmacokinetics, China Pharmaceutical University, Nanjing, China). Maximal plasma concentrations (C_{max}) and the time points at which they occurred (T_{max}) were determined by inspection of the plasma concentration-time profile. The terminal elimination rate constant (λ_z) was determined by linear regression of the terminal portion of the log concentration-time profile. The elimination half-life ($T_{1/2}$) was calculated as $0.693/\lambda_z$. Area under the plasma concentration-time curve (AUC) was determined by trapezoidal rule and extrapolated to infinity by calculation of C_t/λ_z. F=[$AUC_{(0\text{-}t)}$ (T)/$AUC_{(0\text{-}t)}$ (R)]×100%. Log transformed C_{max}, $AUC_{(0\text{-}t)}$, $AUC_{(0\text{-}\infty)}$ were analyzed using ANOVA analysis and Schuirmann two-one sided t-test. T_{max} was analyzed by the non-parametric test and Wilcoxon's test. A P value of less than 0.05 was considered statistically significant. Data were reported as mean ± SD. If the two preparations were bioequivalent, 90% confidence intervals (CI) for test/reference ratios of C_{max}, $AUC_{(0\text{-}t)}$ and $AUC_{(0\text{-}\infty)}$ should fall within the range of 80%-125% [22, 23].

RESULTS

Validation of the assays

Typical chromatograms of spiked ranitidine plasma samples, plasma samples of patients before and after the administration of compound ranitidine preparation are shown in Figure 1. The retention time for ranitidine was 5.1 min. Under the chromatographic condition described, ranitidine and its metabolite were well resolved. Endogenous plasma components did not show any interfering peaks. The assay was linear from 20.0 to 2000.0 μg/L for ranitidine in plasma samples. The mean absolute recovery was about 97.2%, while the intra- and inter-day coefficients of variation and percent error values of the assay method were all lower than 8%. The limit of quantification (LOQ) was found to be 20.0 μg/L, with a precision of less than 20% (n=5) and an accuracy of ±20% (n=5). Ranitidine was stable in plasma samples at different storage conditions: immediately after 3 h at ambient temperature, after perchloric acid treatment and being on the auto-sampler at 4 ℃ for 24 h, after two freeze/thaw cycles and after 1 mo storage at -80 ℃.

Table 1 Pharmacokinetic parameters of ranitidine and bismuth after a single oral dose of compound ranitidine preparation (equivalent to 200 mg of ranitidine and 220 mg of bismuth) in 20 Chinese volunteers (mean ± SD)

Component	Parameters	Test	Reference
Ranitidine	T_{max} (h)	2.3±0.9	2.1±0.9
	C_{max} (mg/L)	0.67±0.21	0.68±0.22
	$T_{1/2}$ (h)	2.8±0.3	3.1±0.4
	$AUC_{(0\text{-}t)}$ (mg/L per hour)	3.1±0.6	3.0±0.7
	$AUC_{(0\text{-}\infty)}$ (mg/L per hour)	3.3±0.6	3.2±0.8
Bismuth	T_{max} (h)	0.50±0.20	0.50±0.20
	C_{max} (μg/L)	11.80±7.36	11.40±6.55
	$T_{1/2}$ (h)	10.2±2.3	13.0±6.9
	$AUC_{(0\text{-}t)}$ (μg/L per hour)	46.65±16.97	47.03±21.49
	$AUC_{(0\text{-}\infty)}$ (μg/L per hour)	55.38±21.27	57.81±23.31

The ICP-MS assay was linear from 0.2-100.0 μg/L for bismuth in plasma samples. The mean absolute recovery of bismuth from quality control samples was about 100.5%, while the intra- and inter-day coefficients of variation and percent error values of the assay method were all lower than 8%. LOQ was found to be 0.2 μg/L, with a precision of less than 10% (n=30) and an accuracy of ±15% (n=30).

The validity study demonstrated that the HPLC assay was reliable for quantification of plasma ranitidine and the ICP-MS assay was reliable for determination of plasma bismuth levels. The specificity, sensitivity, accuracy and precision all met the requirement for PK study.

Concentration-time curves and PK of ranitidine

The average concentration-time curves are shown in Figure 2. The main PK parameters including C_{max}, $AUC_{(0\text{-}t)}$, $AUC_{(0\text{-}\infty)}$, T_{max} and $T_{1/2}$ are given in Table 1. The variation

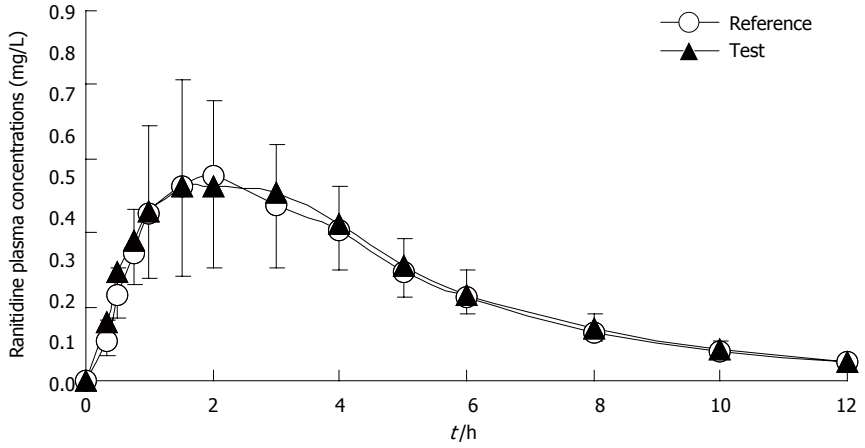


Figure 2 Concentration-time curves of ranitidine in 20 Chinese volunteers after oral administration of two compound preparations. $n = 20$. Mean ± SD. Bars indicate standard deviations (lower bars for test drug and upper bars for reference drug).

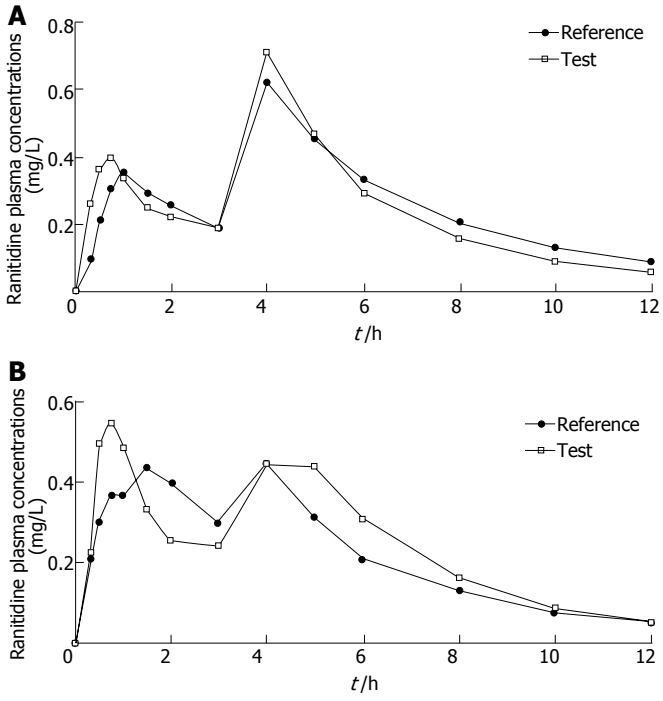


Figure 3 Typical double-peak plasma profiles of ranitidine in two Chinese volunteers (№14 and №16) after oral administration of two compound preparations.

sources of *P* values and 90% CI for the parameter ratios are presented in Table 2. No statistically significant formulation or period effect was encountered, with *P* value greater than 0.05. Inter-subject differences were the main source of variability in log-transformed $AUC_{(0\text{-}t)}$ and $AUC_{(0\text{-}\infty)}$. The 90% CI for the ratio of C_{max} (86.7%-113.6%), $AUC_{(0\text{-}t)}$ (96.0%-113.4%), and $AUC_{(0\text{-}\infty)}$ (95.3%-112.5%) values for the test and reference products was entirely within the FDA acceptable range of 80%-125%. The data indicated that the 2 preparations were bioequivalent with respect to ranitidine. In addition, no significant difference was obtained in T_{max}.

Interestingly, double-peak plasma profiles of ranitidine were observed in some volunteers receiving either test drug or reference drug. Typical double-peak plasma profiles of ranitidine in two volunteers (№ 14 and № 16) after oral administration of two compound preparations are shown in Figure 3.

Table 2 ANOVA for assessment of the drugs, subjects and period effects, and 90% CI for the test/reference ratio of C_{max}, $AUC_{(0\text{-}t)}$ and $AUC_{(0\text{-}\infty)}$, using logarithmic transformed data, after administration of two compound ranitidine preparations (equivalent to 200 mg of ranitidine and 220 mg of bismuth) to 20 Chinese volunteers ($\alpha = 0.05$).

Component	Parameters	ANOVA (*P*-value)			90% CI (%)
		Variation source			
		Drug	Subjects	Period	
Ranitidine	C_{max}	0.9191	0.0686	0.7679	86.7-113.6
	$AUC_{(0\text{-}t)}$	0.3917	0.0153	0.8366	96.0-113.4
	$AUC_{(0\text{-}\infty)}$	0.4733	0.02118	0.9064	95.3-112.5
Bismuth	C_{max}	0.65695	0.51515	0.39551	89.8-120.0
	$AUC_{(0\text{-}t)}$	0.66066	0.11347	0.71245	88.1-124.0
	$AUC_{(0\text{-}\infty)}$	0.75149	0.15804	0.87169	85.3-111.5

Concentration-time curves and PK of bismuth

The average concentration-time curves are shown in Figure 4. The main PK parameters including C_{max}, $AUC_{(0\text{-}t)}$, $AUC_{(0\text{-}\infty)}$, T_{max} and $T_{1/2}$ are given in Table 1. The variation sources of *P* values and 90% CI for the parameter ratios are presented in Table 2. No statistically significant formulation or period effect and inter-subject differences were encountered, with *P* value greater than 0.05. The 90% CI for the ratio of C_{max} (89.8%-120.0%), $AUC_{(0\text{-}t)}$ (88.1%-124.0%), and $AUC_{(0\text{-}\infty)}$ (85.3%-111.5%) values for the test and reference products was also entirely within the FDA acceptable range of 80%-125%. In addition, no significant difference was obtained in T_{max} ($P > 0.05$).

Safety

The two preparations were well tolerated by the volunteers. Unexpected incidents that could have influenced the outcome of the study did not occur. There was no drop-out and all volunteers participating in the study continued to the end. Post-study clinical laboratory tests revealed normal results.

DISCUSSION

The assay methods we developed for ranitidine and bismuth are reliable and applicable in bioavailability study. With respect to HPLC assay for ranitidine, direct

injection of the plasma samples after deproteination using perchloric acid warranted high sensitivity, good accuracy and precision and least time for sample preparation. Most literature on ranitidine determination in plasma involves solid-phase extraction (SPE)[24-26] or liquid-liquid extraction (LLE)[27-29]. However, the methods employing LLE not only consume large time for sample preparation but also give highly variable and relatively low recoveries. The SPE techniques are quite good, but they still involve several steps and need several hundred cartridges. With respect to the ICP-MS assay we developed for bismuth, sample preparation method is more simple and has less matrix effect than the method provided by Mauras Y *et al*[30].

The PK of ranitidine derived from ranitidine bismuth citrate is consistent with observations for ranitidine administered alone[31]. However, literature on PK of ranitidine derived from compound preparation is not yet available. Wen *et al*[32] showed that. C_{max} and $AUC_{(0-\infty)}$ derived from a single 300 mg dose of ranitidine alone are 0.91 mg/L and 4.78 mg/L per hour, respectively. However, when 300 mg colloidal bismuth sub-citrate capsules are co-administered, the two parameter values are significantly decreased by 41.8% and 33.7%, respectively. No significant differences have been observed with respect to T_{max} and $T_{1/2}$ under the two different circumstances, suggesting that ranitidine granules are partially enwrapped by colloidal precipitation as presented by Wen *et al*[32], which can explain the loss in ranitidine absorption. Ranitidine is characterized by a dose-proportional PK. So C_{max} and $AUC_{(0-\infty)}$ for ranitidine mono-preparations are both standardized to dose (200 mg), ranging 0.60-1.20 mg/L and 3.0-4.5 mg/L per hour, respectively[16-19,33]. The mean C_{max} and $AUC_{(0-\infty)}$ values for ranitidine derived from combined formulation in our study were 0.67 mg/L and 3.1 mg/L per hour, respectively, falling within the above range for ranitidine administered alone. If significant loss exists at absorption phase of ranitidine derived from test compound formulation and the decreased percentage is similar with the results of Wen *et al*[32]. C_{max} and $AUC_{(0-\infty)}$ values for ranitidine mono-preparation would be estimated to be 1.15 mg/L and 4.7 mg/L per hour, respectively, still being within/or near the mean range. Thus, the possible effects of bismuth potassium citrate on ranitidine absorption could not be excluded, thus more relative studies are needed to confirm the results presented by Wen *et al*[32]. Moreover, it would be useful to investigate the difference between the bioavailability of ranitidine in the compound preparation relative to the administration of two mono-preparations that are ingested simultaneously, although compound preparation has advantages such as more convenience in administration and better medication compliance in ulcer patients. However, the main purpose of this study was to confirm whether the two compound preparations can be prescribed interchangeably by bioequivalence evaluation.

The PK of oral drugs exhibiting double peaks cannot be adequately described by conventional compartmental models[34]. So we used non-compartmental method. The double-peak phenomenon of ranitidine plasma profile in several volunteers is consistent with previous reports[34,35,38]. However, this PK profile has not been revealed in Chinese. The proposal mechanism responsible for the existence of secondary peaks, presented by Yin *et al*[34] and Schaiquevich *et al*[35], includes enterohepatic recirculation and the existence of multiple sites of absorption along the gastrointestinal tract.

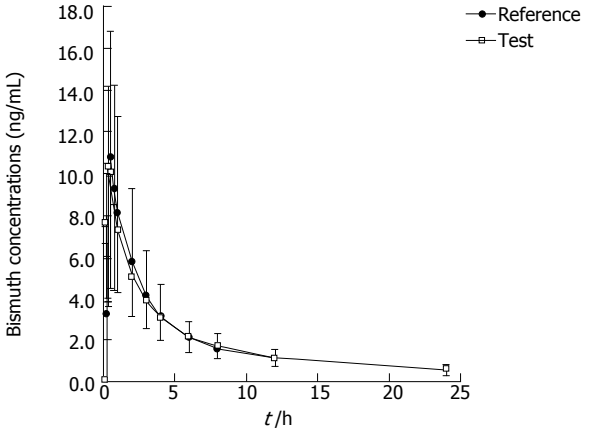


Figure 4 Concentration-time curves of bismuth in 20 Chinese volunteers after oral administration of two compound preparations. $n = 20$, mean ± SD. Bars indicate standard deviations (lower bars for test drug and upper bars for reference drug).

The mean T_{max} value for bismuth derived from compound preparations in this study was 0.50 h, indicating that bismuth is rapidly absorbed by Chinese volunteers. The result is similar with those presented by Nwokolo *et al*[13] and Benet *et al*[14]. Peak bismuth plasma concentrations in our study ranged 3.24 μg/L-27.50 μg/ L, the mean C_{max} value was 11.60 μg/L, suggesting that bismuth neurotoxicity is not associated with steady-state concentrations of 50-100 μg/L. The mean terminal half-lives for bismuth derived from the test compound preparation (equivalent to 220 mg bismuth) in this study was 10.2 h, which is nearly similar with the mean values of 9.1 h derived from a single dose of 350 mg ranitidine bismuth citrate capsules for 10 Chinese health volunteers[36]. Benet *et al*[14] considered that the intermediate half-life of 5-11 d derived from colloidal bismuth sub-citrate represents most of the clearance and elimination. Moreover, the plasma terminal half-lives averaging 21 d are derived from oral doses of 800 mg ranitidine bismuth citrate, twice daily for 28 d[31]. These different results are suggestive of possible ethnic differences in bismuth elimination or indicate that multiple-dose administration of bismuth may result in its prolonged elimination. In this way, further investigations should be carried out.

A placebo-controlled crossover study showed that daily oral administration of 40 mg omeprazole for 1 wk increases the systemic availability of 240 mg bismuth derived from tripotassium dicitrate bismuthate. The explanation by Treiber *et al*[37] is that absorption of bismuth may be dependent on intragastric pH which is elevated by omeprazole. However, the mean $AUC_{(0-t)}$ of bismuth derived from test compound preparation (equivalent to 200 mg of ranitidine and 220 mg of bismuth) in our study was 46.65 μg/L per hour, which is nearly similar with the value of 46 μg/L per hour derived from mono-preparation of tripotassium dicitrate bismuthate (equivalent to 220 mg of bismuth)[37]. Thus, additional experiments are also

needed to address whether ranitidine increases the systemic availability of bismuth.

The most important objective of bioequivalence testing is to assure the safety and efficacy of generic formulations. Two drugs are considered to be bioequivalent and thus therapeutically equivalent if they are pharmaceutical equivalents (i.e., similar dosage forms made, perhaps by different manufacturers) or pharmaceutical alternatives (i.e., different dosage forms) and if their rates (C_{max}) and extents of absorption (AUC) do not show a significant difference when administered at the same molar dose of the therapeutic moiety under similar experimental conditions[39]. Interpretation of T_{max} is difficult in some oral bioequivalence studies, because of the occurrence of multiple peaks. Comparison between the formulations is based on the overall C_{max}, T_{max}, and AUC. Therefore, the presence or absence of a double peak can not affect the accuracy of measurements[40].

It is generally accepted that the standard equivalence range is 0.8-1.25 for basic pharmacokinetic characteristics, such as AUC and C_{max}[22,23]. For ranitidine derived from the two compound preparations in this study, no significant difference was obtained in T_{max} ($P>0.05$) and 90% CI for the test/reference ratio of C_{max}, $AUC_{(0\text{-}t)}$ and $AUC_{(0\text{-}\infty)}$ was found within the range 80%-125%. Therefore the two compound preparations can be expected to be equally effective with respect to the action of ranitidine. To support equal clinical efficacy of compound preparations, it is necessary to show the equivalent gastric availability (local effect) of the bismuth component. Its effect for a large part is determined by the pharmaceutical form in which this component is delivered. Coghill *et al*[20] have developed a novel means of comparing the bioequivalence of the two preparations of tripotassium dicitrate bismuthate (De-Noltab) in patients attending a gastroscopy clinic by examining duodenal biopsies with electron microscopy. However, this method is invasive, thus lacking applicability. We performed a comparative bioavailability study to reflect the equal gastric availability indirectly. The results have confirmed the equivalent rates and extents of absorption of bismuth derived from the two compound preparations. Thus, we consider that these two compound preparations have equal clinical efficacy with respect to bismuth.

In conclusion, the 2 compound preparations are bioequivalent with respect to both ranitidine and bismuth and can be prescribed interchangeably.

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BASIC RESEARCH

Modulatory effects of *Azadirachta indica* on benzo(a)pyrene-induced forestomach tumorigenesis in mice

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Abstract

AIM: To evaluate the chemopreventive effects of aqueous *Azadirachta indica* (*A indica*) leaf extract (AAILE) against benzo(a)pyrene [B(a)P]-induced forestomach tumorigenesis in Balb/c mice.

METHODS: Female Balb/c mice were divided into four groups of 10-12 animals each. For induction of forestomach tumors, starting from d 14 of the experiment, mice of B(a)P and B(a)P+*A indica* groups were given intra-gastric instillations of B(a)P (40 mg/kg), twice a week for four weeks. Mice of *A indica* and B(a)P+*A indica* groups were orally administered with AAILE (100 mg/kg), two weeks prior to B(a)P instillations till the end of the experiment. After 22 wk of the first B(a)P instillation, mice were sacrificed and the forestomachs were analyzed for development of tumors, scanning electron microscopy (SEM) and histopathology.

RESULTS: Tumor incidence was observed to be 100% in mice that received only B(a)P. However, treatment with AAILE reduced the tumor incidence by 58.4% as observed in mice of B(a)P+*A indica* group when compared to that of B(a)P group. Similarly, the tumor burden and multiplicity were seen to decrease by 87.3% and 69.6% respectively in mice of B(a)P+*A indica* group when compared to those of B(a)P group. Scanning electron microscopy analysis showed that AAILE treatment itself did not cause any abnormalities on the surface architecture of forestomach epithelium. In tumorous forestomach, surface disruption was observed. Over the forestomach tumors of B(a)P group of mice certain rounded structures were seen in addition to closely placed tongue-shaped squamous cells. Interestingly, these rounded structures were not observed in B(a)P + *A indica* group of mice. Histopathalogically, the tumors were identical and diagnosed to be papillomas. Mice from control and *A indica* groups of mice did not develop any forestomach tumors and showed normal histo-architecture.

CONCLUSION: The present data suggest that *A indica* exerts chemopreventive effects against B(a)P-induced forestomach tumors in murine model. Because of lack of toxicity and ubiquitous bioavailability, *A indica* may play a promising role in future drug discovery and development as far as chemoprevention of cancer is concerned.

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Key words: *Azadirachta indica*; Benzo(a)pyrene; Chemoprevention; Histopathology; Scanning electron microscopy

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INTRODUCTION

Recent estimates show that gastric cancer is the fourth most frequent type of cancer and the second most prevalent cause of cancer deaths worldwide[1]. Human beings are exposed to a multitude of carcinogens in their environment, and majority of the cancers are considered to be chemically-induced[2]. Polycyclic aromatic hydrocarbons (PAHs) are environmental carcinogens present in the atmosphere from combustion sources such as diesel exhaust, cigarette smoke, residential heating processes, refuse burning, industrial coke production, volcanic eruption, and oil contamination by effluents and oil spills[3]. They can also be generated by the pyrolysis of amino acids, fatty acids and carbohydrates during cooking process[4]. After inhalation, ingestion is the second most important exposure route for PAHs to enter the human body[5]. Stomach is the organ where engulfed/ingested materials come into prolonged contact with gastro-intestinal (GI) mucosa[6]. So it is quite reasonable to assume that stomach has high risk of developing cancer from carcinogens entering the GI tract. Benzo(a)pyrene

[B(a)P], a potent pro-carcinogen employed in initiating stomach cancer, is the prototypical and best characterized member of PAHs family of chemical carcinogens[7]. It has been shown to induce tumors of skin, lung, mammary glands and forestomach tissues of various experimental animals[8]. Since B(a)P is an omnipresent environmental pollutant and is believed to be a risk factor for human chemical carcinogenesis, it is important to identify the naturally occurring/synthetic agents that could modulate B(a)P-induced tumorigenesis[9].

In experimental studies as well as pre-clinical systems, several drugs have been discovered to combat cancer, but are not much in use because of their undesirable toxic side effects[10]. Moreover, most of the synthetic chemotherapeutic drugs available today are cytotoxic, immuno-supressants and may cause a variety of side effects in the normal organs of the body[11]. Prevention is an effective strategy to control the incidence of cancer. Recent progress in molecular biology and pharmacology enhances the likelihood that cancer prevention will increasingly rely on chemoprevention[12]. The later is a means of cancer control, in which the occurrence of disease as a consequence of exposure to carcinogen can be slowed, blocked or reversed by the administration of one or more naturally occurring or synthetic compounds[13]. Chemoprevention also deals with the chemotherapy of pre-cancerous lesions, which are called pre-invasive neoplasia, dysplasia or intra-epithelial neoplasia depending on the organ system[14]. It has been observed that the synthetic chemopreventive agents produce toxic side effects, which have limited their extensive use[15]. Plants, vegetables as well as medicinal herbs used in the folk and traditional system of medicine have been accepted currently as one of the main source of cancer chemopreventive drug discovery and development[16]. Ayurveda, the Indian traditional system of medicine, which dates back many centuries, uses many herbal extracts to cure a variety of diseases including cancer[17]. There is a growing interest in evaluation of chemopreventive efficacy of plants used in Ayurveda and one of such plants is *A indica* commonly called Neem.

A indica leaf extract has been reported to be non-toxic, non-mutagenic and possesses immuno-stimulant, hepato-protective, anti-oxidant, anti-genotoxic and anti-cancer activities[18]. The anticancer activities of *A indica* leaf extract have attracted much of the attention of research community in last few years. Subapriya *et al*[19] have observed chemopreventive activity of ethanolic *A indica* leaf extract against N-methyl-N-nitro-N-nitroso-guanidine-induced gastric carcinogenesis in rats. Dasgupta *et al*[9] have reported the chemopreventive effects of ethanolic *A indica* leaf extract against DMBA-induced TPA promoted skin and B(a)P-induced forestomach papillomagenesis. AAILE has been shown to ameliorate 4-nitroquinoline 1-oxide-induced oral tumorigenesis in male Wister rats[20] and 7-12 dimethylbenz(a)anthracine (DMBA)-induced buccal pouch carcinogenesis in Syrian hamsters[21]. Inhibitory effect of AAILE against DMBA-induced skin carcinogenesis in mice has recently been reported from our research laboratory[22].

Keeping in view the above mentioned reports, the present study was designed to evaluate the modulatory effects of AAILE, if any, on B(a)P-induced forestomach tumorigenesis in Balb/c mice. Tumor incidence, burden and multiplicity were taken as an index for chemopreventive tumor response. In addition to histopathology, scanning electron microscopy (SEM) of forestomach tumors was also done in an attempt to examine the surface morphology of tumorous forestomach. To the best of our knowledge the SEM of tumorous forestomach of Balb/c mice has not been reported yet.

MATERIALS AND METHODS

Materials

B(a)P was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Olive oil used as a vehicle for B(a)P, and all other chemicals utilized were obtained from reputed local firms (India) and were of the highest purity grade. Capsules containing 100 mg of spray dried AAILE were obtained from a recognized herbal company (Dabur India Ltd). The contents of the capsules were dissolved in double-distilled water immediately before its oral administration to the mice in order to attain the required doses as described by Balasenthil *et al*[21]. The solution obtained after dissolving AAILE was covered with dark paper in order to protect the same from direct exposure to light.

Animal experiment

Random-bred virgin female Balb/c mice (6-8 wk old) procured from Central Animal House, Panjab University, Chandigarh were housed in polypropylene cages bedded with sterilized rice husk. All the mice were allowed to have free access to clean drinking water and standard animal pellet diet formulated on scientific backgrounds (Ashirwad Industries, Tirpari, Kharar, Distt. Ropar, Punjab) throughout the experiment. All the experimental protocols were approved by the Institutional Ethics Committee (Panjab University, Chandigarh, India) and conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals. Before the commencement of various treatments, mice were kept for acclimatization to experimental conditions for one week and the final day of their acclimatization period was considered as day 0 of the experiment. Mice were randomly divided into four groups (10-12 mice per group) namely control group, B(a)P group, *A indica* group and B(a)P + *A indica* group. Mice of B(a)P and B(a)P + *A indica* groups were given B(a)P for induction of forestomach tumor as described by Wattenberg *et al*[23], with minor modification. Briefly, each mouse received intra-gastric instillations of 40 mg B(a)P/kg in 0.2 mL olive oil, twice a week for four weeks from d 14 to d 42 of the experiment (a total of eight B(a)P instillations per mouse). Also, the animals of control and *A indica* groups received 0.2 mL of only olive oil in the same schedule. Mice of *A indica* and B(a)P + *A indica* groups were orally administered (using blunt tipped canula and a syringe) with AAILE at a dose of 100 mg/ kg thrice a week on alternate days throughout the experiment as described by Balasenthil

et al[21]. Mice from all the groups were observed for alterations in body weight and diet and water consumption. After 22 wk of the first B(a)P instillation, mice from all the groups were sacrificed by cervical dislocation under light ether anesthesia. The stomachs were carefully removed and processed for different parameters.

Chemopreventive tumor response

The stomachs were kept in expanded state by injecting them with 40 g/L buffered formaldehyde solution. Twenty-four hours later, the stomachs were split longitudinally and the forestomachs were carefully observed under the dissecting microscope for the presence of tumors. Forestomach tumors (1 mm in diameter) were counted/recorded for each mouse. The chemopreventive tumor response was assessed on the basis of tumor incidence, burden and multiplicity that were calculated as follows: tumor incidence: percentage number of animals having tumors; tumor burden: total number of tumors counted/total number of mice; tumor multiplicity: total number of tumors counted /number of tumor bearing mice.

Scanning electron microscopy

For SEM studies, the forestomachs from all the groups of mice were fixed in 4% gluteraldehyde prepared in 200 mmol/L phosphate buffer (pH 7.2) for two hours followed by two washings in phosphate buffer. After this, the tissues were dehydrated in ascending grades of acetone, i.e. 30%, 50%, 70%, 90% and absolute acetone (for 30 min per grade). Tissues were then immersed in a mixture of acetone and amyl acetate (1:1, V/V) followed by amyl acetate alone (30 min each). Following this, the tissues were subjected to critical point drying (CPD) to get rid of remaining water. After CPD, the tissues were pasted on metallic stubs and further trimmed as per requirement. The stubs were placed inside the sputter for gold coating (15 min). These were then viewed under scanning electron microscope of Jeol 2601, installed at Regional Sophisticated Instrumentation Center, Panjab University, Chandigarh.

Histopathology

Formalin fixed tissues were processed for HE staining using the conventional laboratory procedure. Briefly, the tissues were dehydrated through ascending grades of alcohol, cleared in benzene and embedded in low melting point paraffin. Sections of 5 micrometer thick were cut, placed serially on clean glass slides and then deparaffinised through descending grades of alcohol. Three contiguous sections were made from each forestomach tissue and stained with HE for evaluation under light microscope. The slides were coded, so that the particular sample identity was not known while performing assessment.

Statistical analysis

Statistical significance of differences for tumor burden and multiplicity was analyzed by Student's *t*-test. Comparison was carried out between B(a)P and B(a)P + *A indica* groups.

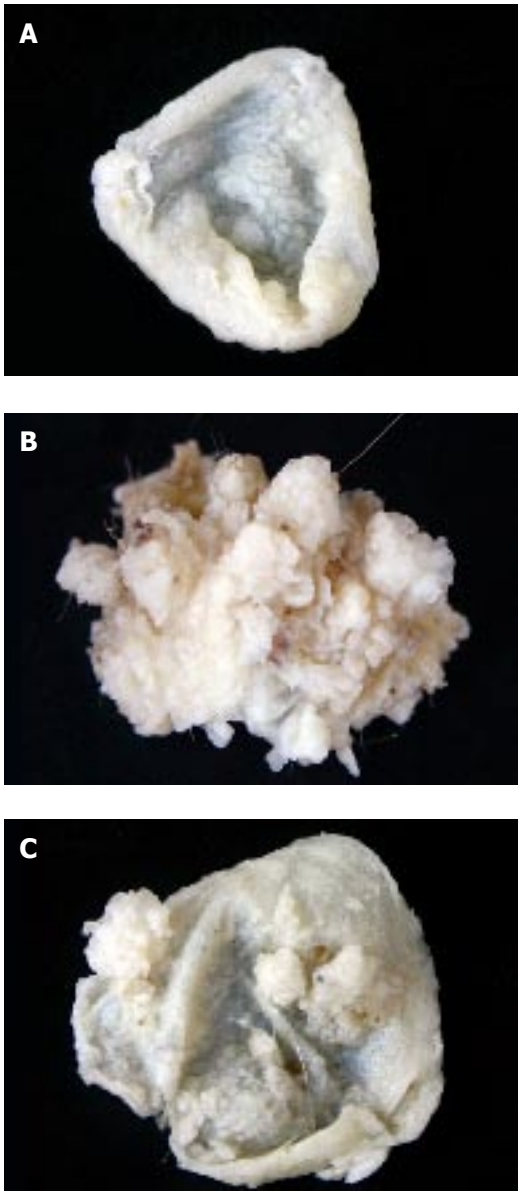


Figure 1 Gross anatomy of forestomach showing normal surface (**A**), tumorous surface in mice that received only B(a)P (**B**), and lesser number of tumors in mice that received AAILE along with B(a)P (**C**).

RESULTS

In the present study, it was noticed that the diet and water consumption by the mice in all the groups studied was approximately same during the entire experimental span (data not shown). Mice from all the groups showed normal body growth and gained weights when compared to their respective initial body weights (Table 1). It was observed that the mice from B(a)P and B(a)P + *A indica* groups developed forestomach tumors, whereas none of the mice from control & *A indica* groups developed forestomach tumors (Figures 1A-C). Tumor incidence was observed to be 100% in B(a)P group of mice. However, the tumor incidence was found to be 41.6% in the mice of B(a)P + *A indica* group, i.e. the mice that received AAILE treatments along with B(a)P instillations. This experiment clearly

Table 1 Modulatory effects of AAILE on B(a)P-induced forestomach tumorigenesis in Balb/c mice

Groups	Body mass (g)		Tumor incidence	Tumor burden	Tumor multiplicity	No. of Tumors (Min-Max)
	Initial	Final				
Control	22.1±1.51	27.6±1.64	0 % (0/10)	-	-	-
B(a)P	22.6±1.68	26.5±2.24	100 % (12/12)	7.25±2.80	7.25±2.80	5-14
A indica	22.4±1.78	27.8±1.94	0 % (0/10)	-	-	-
B(a)P+*A indica*	22.3±1.64	27.1±1.64	41.6% (5/12)	0.92±1.24[b]	2.20±0.84[b]	0-3

[b]$P<0.001$ *vs* B(a)P.

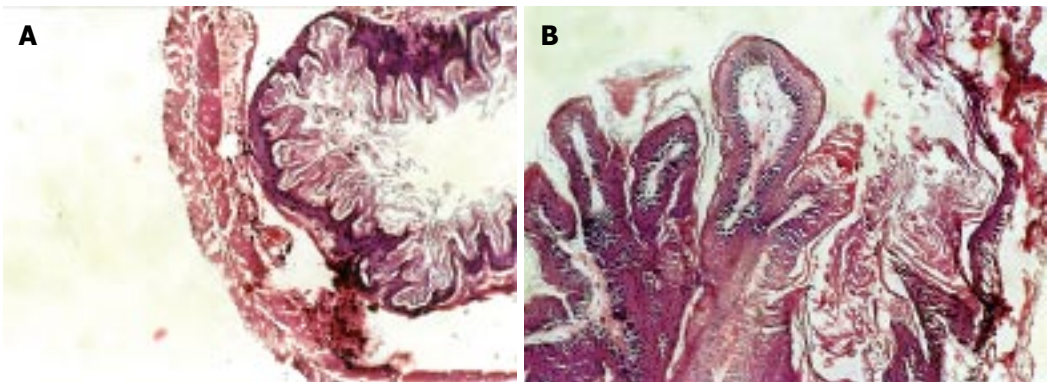


Figure 2 Histo-micrograph of forestomach. **A**: Control mice showing squamous mucosa, sub-mucosa and muscular layers X 20; **B**: Papillary projections, disrupted submucosa and muscular layers X 20.

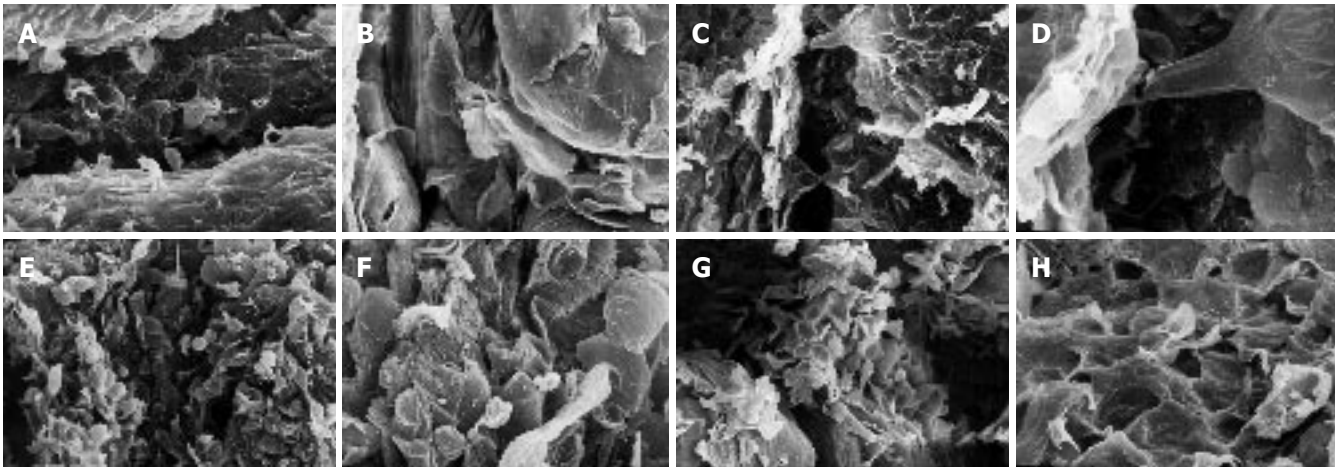


Figure 3 SEM of forestomach of mice. **A**: Control mice showing grooves and ridges X 200; **B**: Control mice showing tongue-shaped squamous epithelium X 600; **C**: Mice treated with only AAILE showing grooves and ridges X 200; **D**: Mice treated with only AAILE showing squamous epithelial cells X 600; **E**: Mice received only B(a)P showing ruptured surface X 200; **F**: Mice received only B(a)P showing certain rounded structures in addition to tongue-shaped squamous epithelial cells X 600; **G**: Mice received AAILE along with B(a)P instillations showing surface rupturing X 200; **H**: Mice received AAILE along with B(a)P instillations showing tongue shaped squamous epithelial cells X 600.

demonstrated that AAILE potentially reduced the tumor incidence by 58.4 %. The tumor burden was observed to decrease significantly (approximately 7.88 fold) upon AAILE treatment when the mice of B(a)P+*A indica* group were compared to that of B(a)P group. Similarly, the tumor multiplicity was observed to decrease (approximately 2.59 folds) upon AAILE treatment as seen in mice of B(a)P + *A indica* group when compared to that of B(a)P group (Table 1). In another similar investigation, Dasgupta *et al*[9] reported that the administration of ethanolic neem leaf could reduce the tumor incidence by 39% during B(a)P-induced forestomach tumorigenesis in Swiss albino mice.

Forestomach of control group as well as *A indica* group of mice showed normal surface morphology with distinct grooves and ridges (Figures 3A and 3C). Mucous sheath at certain places and tongue-shaped keratinized squamous epithelial cells arranged in layers like tiles were observed[24] (Figures 3B and 3D). These observations suggested that AAILE treatment itself could not cause any abnormalities over the epithelium. In forestomach of tumor bearing mice, surface disruption was observed at the sites of tumor development (Figures 3E and 3G). In forestomach tumors of the mice that received only B(a)P, within the closely placed squamous epithelial cells, in addition to tongue-shaped cells certain rounded to irregularly shaped structures were seen (Figure 3F). The observed rounded structures could be transformations

of tumorous cells. However in tumors of the mice that received AAILE treatments along with B(a)P instillations, such structures were not seen (Figure 3H). Their absence in the forestomach tumors of B(a)P + *A indica* group of mice could be attributed to the different cytotoxic effects of the phytochemical components present in AAILE.

Histopathological analysis showed that forestomach of mice from control and *A indica* groups depicted normal histoarchitecture with distinct muscular layers, sumucosa and squamous mucosa (Figure 2A). The tumors developed in B(a)P and B(a)P + *A indica* groups were observed to be similar and diagnosed as papillomas with endophytic growth in the form of papillary projections having fibro-vascular core lined by stratified squamous epithelium (Figure 2B). Though the tumors developed in B(a)P as well as in B(a)P + *A indica* were similar histopathologically, our observations clearly suggested the chemopreventive effects of AAILE against B(a)P induced forestomach tumorigenesis.

DISCUSSION

Stomach cancer is the second most prevalent malignancy in the world and chemoprevention has evolved as an effective strategy to combat this dreadful disease[25]. Fruits, vegetables, vitamins, common beverages and several medicinal plants/herbs with diversified pharmacological properties have been shown to be a rich source of cancer chemopreventive agents[26]. *A indica*, one of the most versatile traditional medicinal plants, has been known to possess a wide spectrum of medicinal properties and is used in Ayurveda to cure several ailments including cancer. In the present piece of research work, an attempt was made to evaluate the modulatory effects of AAILE against B(a)P-induced forestomach tumorigenesis.

B(a)P, an extremely potent pro-carcinogen, is metabolized by biotransformation enzymes to a variety of metabolites that are responsible for initiating tumorigenesis[27]. Biotransformation enzymes have broadly been divided into two categories namely phase-I and phase-II biotransformation enzymes. The former constitutes cytochrome P-450 based mono-oxygenase system which is responsible for initiating conversion of pro-carcinogens to several of their metabolites including ultimate carcinogens. Glutathione-S-transferase (GST) is a major phase II detoxifying enzyme that primarily functions in catalyzing the active carcinogenic metabolites to endogenous ligand-reduced glutathione (GSH) favoring their elimination from the body of the organisms[28]. The balance between the phase I carcinogen-activating enzymes and the phase II detoxifying enzymes is critical to determining an individual's risk for cancer[29]. There is substantial evidence that chemopreventive agents including medicinal plants exert their anti-carcinogenic effects by modulation of phase I and phase II xenobiotic biotransformation enzymes[30]. A significant reduction in the activity of cytochrome P450 and cytochrome b_5 (phase I enzymes) in hepatic tissue of mice upon AAILE treatment has recently been reported from our laboratory[31]. Ethanolic *A indica* leaf extract can exert down-regulatory effect on the activity of cytochrome P450 and cytochrome b_5 in some organs like liver, kidney and forestomach of mice[9]. Maintaining rats on diets containing neem flowers has been reported to decrease the activity of cytochrome P-450 in hepatic tissue that have been correlated with inhibition of aflatoxin B-1- and DMBA-induced liver and mammary gland carcinogenesis respectively[32]. *A indica* leaf extract can also enhance the activity of GST as well as GSH contents (phase II components) in different tissues of a number of experimental animal models[9,21,31]. These observations suggest that the AAILE treatment decreases the metabolic activation with simultaneous increase in the detoxification of B(a)P. Therefore the chemopreventive effects of AAILE observed in the present study could be ascribed, at least in part, to its ability to modulate certain components of carcinogen biotransformation system.

Reactive oxygen species (ROS) are widely generated in biological systems either by normal metabolic pathways or as a consequence of exposure to chemical carcinogens leading to oxidative stress that may further result in membrane dysfunction, protein inactivation, DNA damage ultimately contributing to the process of carcinogenesis[33]. For protection against the deleterious effects of these ROS, organisms have developed a sophisticated antioxidant defense system which has enzymatic as well as non-enzymatic components. The antioxidant defense system includes enzymes like glutathione peroxidase (GPx), catalase, glutathione reductase (GR), GST and superoxide dismutase (SOD), whereas non-enzymatic components include non-protein thiol GSH, uric acid, some trace metals and certain vitamins such as ascorbic acid and alpha-tocopherol, *etc*[34]. *A indica* leaf extract has been shown to enhance the activities of all the above mentioned antioxidant defense enzymes and GSH contents in various tissues of mice and rats[9,19]. Increased antioxidant defense upon AAILE treatment could lower the ROS-mediated damage at the initiation as well as progression/promotion phase of the process of tumorigenesis.

Nimbin is the first phyto-chemical compound that has been isolated from *A indica* seed oil[35] and now approximately 140 different phytochemicals have been identified and isolated from various parts of this plant[36]. The anti-carcinogenic effects of this tree are also attributed to its antioxidant phytochemicals such as flavonoids[37]. Quercetin and kaemferol are the flavonoids present in neem leaf that have been documented to retard carcinogenesis at initiation, as well as promotion phases of carcinogenesis by virtue of their radical scavenging properties[38,39]. Although we did not carry out quantitation and characterization of individual components from AAILE in this study, the contribution of antioxidant phytochemicals of AAILE in reducing the extent of tumorigenesis can not be ruled out.

Yadav *et al*[40] showed that mitotic activity can be inhibited by *A indica* leaf extract. Baral *et al*[41] reported that the growth of Ehrlich carcinoma and B16 melanoma cells is reduced significantly after administration of *A indica* leaf extract. Limonin 17β-D-glucopyranoside, a limonoid found in neem, can inhibit DMBA-induced oral carcinogenesis[42]. Azadirone 1, a limonoid constituent of *A indica* possesses cytotoxic activity against breast, melanoma and prostate cancer cell lines[43]. Cytotoxicity of azadirachtin A has been reported in human glioblastoma cell lines by

Akudugu *et al*[44]. Nimbolide and 28-deoxonimbolide have been identified as cytotoxic constituents of *A indica* leaves[45]. Intra-peritoneal administration of some isolated polysaccharides from *A indica* is active against sarcoma -180 ascites tumor cells in mice[46]. In another investigation Petit *et al*[47] reported that some active components of *A indica* show cytotoxic effects against murine P_{-388} lymphocytic leukemia. In the present study, since AAILE was given throughout the experiment, the cytotoxic effects of its active constituents against pre-cancerous / tumorous cells could not be denied.

In *in-vivo* experiments, it has been observed that *A indica* leaf extract treatment does not alter the activity of cellular lactate dehydrogenase (an indicator of cell damage) in the hepatic tissues, suggesting that the leaf extract itself does not exert any adverse effects[9]. Also, it is generally believed that the crude therapeutic preparations are more effective and less toxic than the isolated active components[48] or their respective synthetic counterparts because they contain the total family of medicinal compounds (known and unknown) just as they are found in their natural source and hence offer less risk of side effects. In crude preparations, perhaps, the other components present in addition to the active components may affect the effects of the active components. These known and unknown components might act as synergists for the therapeutic effects and antagonists for the side effects of the active components as well as the other toxic components in the crude preparation[49].

The present data together with reports available in literature, suggest that *A indica* has immense chemopreventive potentials as far as B(a)P-induced forestomach tumorigenesis is concerned. *A indica*-based therapeutic preparations have already been used in the Indian traditional system of medicine for several ailments. Previous experimental investigations along with the present study support the rational use of these preparations for chemoprevention of certain types of cancers. Because of lack of toxicity and ubiquitous distribution of this plant in nature, *A indica* seems to be a valuable plant source for use in modern chemopreventive drug discovery and development.

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CLINICAL RESEARCH

Efficacy and safety of ecabet sodium on functional dyspepsia: A prospective, double-blinded, randomized, multi-center controlled trial

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Abstract

AIM: To compare ecabet sodium and cimetidine in relieving symptoms of functional dyspepsia.

METHODS: We performed a multi-center, prospective, randomized, double-blinded controlled trial to compare the clinical efficacy of ecabet sodium and cimetidine in patients with functional dyspepsia. Two-hundred and seventy-two patients with dyspeptic symptoms fulfilling the Rome-II criteria were enrolled from 7 centers. In the study group (115 patients), 1.5 g ecabet sodium was given twice a day. In the control group (121 patients), 400 mg cimetidine was given twice a day. Symptoms and parameters of quality of life were analyzed at baseline, 3, 14, and 28 d after initiating the treatment.

RESULTS: Two-hundred and thirty-six patients completed the clinical trial. After 4 wk of treatment, the rates of improvement in patients with dyspeptic symptoms were not different between two groups (77.4% in the ecabet group and 79.3% in the cimetidine group, respectively, $P > 0.05$). Likewise, the rates of symptomatic improvement were not different at 3 d and 14 d. The parameters of quality of life did not change significantly during the study period in both groups. There was no clinically significant adverse event in both groups.

CONCLUSION: In patients with functional dyspepsia, ecabet sodium has similar clinical efficacy with cimetidine.

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Key words: Functional dyspepsia; Ecabet sodium; Cimetidine

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INTRODUCTION

Functional dyspepsia is defined as persistent or recurrent abdominal pain or abdominal discomfort centered in the upper abdomen which cannot be explained by structural or biological abnormalities[1,2]. Discomfort refers to unpleasant sensations that the subject does not interpret as pain and may be characterized by upper abdominal fullness, early satiety, bloating, belching, or nausea. For the diagnosis of functional dyspepsia, there should be no relationship between dyspeptic symptoms and bowel movements (i.e., irritable bowel syndrome does not explain the symptoms), and patients with predominant heartburn should be excluded.

At present, the pathophysiology of functional dyspepsia is not fully elucidated. Several mechanisms have been suggested to explain the development of dyspeptic symptoms, including delayed gastric emptying[3], impaired gastric accommodation to a meal[4], hypersensitivity to gastric distention[5,6], *Helicobacter pylori* (*H pylori*) infection[7], and central or peripheral nervous system dysfunction[8].

However, there is growing evidence that functional dyspepsia is in fact a very heterogeneous disorder and different subgroups can be identified based on different demographic, clinical, and pathophysiologic features[2].

Pharmacological treatment for patients with functional dyspepsia remains unsatisfactory. The results of studies with H_2-receptor antagonists have been conflicting[9,10]. Proton pump inhibitors appear to be superior to placebo, but not in dysmotility-like dyspepsia[11]. Cisapride has also been demonstrated to be effective[12], but it has been reported that cisapride is not superior to placebo[13]. Therefore, it seems likely that acid-suppressing agents and prokinetic agents are, at best, only effective in some subgroups of dyspeptic patients.

Ecabet sodium (ES), a 12-sulfodehydroabietic acid monosodium salt, is a novel non-systemic anti-ulcer agent[14-16] and belongs to the category of gastroprotective agents[17]. The utility of ES in clinical setting has been demonstrated in various clinical trials for patients with peptic ulcer, ulcerative proctosigmoiditis, and *H pylori* infection[18-21]. In this prospective randomized controlled study, we attempted to assess the efficacy and safety of ES in patients with functional dyspepsia.

MATERIALS AND METHODS

Patients

From October 2003 to September 2004, this trial was performed at 7 centers in Korea. A total of 272 patients with functional dyspepsia according to Rome-II criteria were enrolled. Upper gastrointestinal endoscopy was performed to exclude a structural cause of the symptoms. All patients understood the purpose and method of this study and agreed to this clinical trial with written informed consent.

Exclusion criteria included age under 18 years or over 75 years, pregnancy or lactation, regular use of steroids or NSAIDs, an experience in administration of any drug for treating the subject disease, including gastric acid secretion inhibitors (proton pump inhibitor, H2-blocker, etc.) within 4 wk of enrollment. Patients demonstrating gastric ulcer or duodenal ulcer on upper gastrointestinal endoscopy, were excluded from this study. Other exclusion criteria included gastroesophageal reflux disease with typical symptoms like heartburn or acid regurgitation, malignant diseases, and serious hepatic, cardiovascular, renal, or hematological diseases. Patients who were unable to answer the visual analogue scale question due to poor intellectual power were also excluded.

Methods

A prospective, double-blinded, randomized, comparative trial was performed in 7 centers. Patients were randomized to receive either ecabet sodium (1.5 g p.o, bid) or cimetidine (400 mg p.o, bid) for 4 wk. All medications were given within 30 minutes after breakfast and just before going to bed.

At screening, upper gastrointestinal endoscopy, complete blood cell counts, blood chemistries and serology for *H pylori* infection were performed. If the result of upper gastrointestional endoscopy within 3 mo of the enrollment was not significant, repeat endoscopic examination was not performed. At the beginning of the study, patients were required to give written informed consent and to answer the questionnaire for the evaluation of the quality of life and for individual dyspeptic symptoms. Assessment of the quality of life using the same questionnaire was repeated at the end of the treatment.

The symptoms were evaluated 4 times (baseline, 3, 14, and 28 d) using index of dyspepsia symptoms-Koreans (IDS-K) questionnaire validated in Korea[22]. This questionnaire format was designed to evaluate 12 typical upper gastrointestinal symptoms using 10-cm visual analogue scale. In addition, patients were asked to judge the global improvement of symptoms as significant improvement, somewhat improvement, no change, somewhat worsen, and severely worsen. In order to measure the overall efficacy of the medication, treatment effect was categorized into 2 groups. Significant improvement and somewhat improvement were judged as improved, while no change, somewhat worsen and severely worsen were judged as not improved.

For the evaluation of the quality of life, FD-QoL questionnaire was completed in coordinator's interview before and at the end of the treatment. The questionnaire was consisted of 21 items of 4 dimensions (5 questions for the eating status, 4 questions for the liveliness status, 6 questions for the psychologic status, and 6 questions for the role-functioning status). Higher scores indicated better quality of life. The sum of scores was calculated and compared to assess the quality of life before and after the treatment. Safety of the medication was evaluated by the assessment of significant adverse events. At the end of the treatment, complete blood cell counts and blood chemistries were repeated.

Statistical analysis

Efficacy data analyses were performed based on per-protocol analysis set. Clinical characteristics of target patients were summarized with technical statistics. Consecutive variables were described with mean ± SD and the categorical variables with proportion. With respect to the global improvement of symptoms, effective group and non-effective group were classified and compared using χ^2-test. With respect to the quality of life, a paired *t*-test was used to compare the difference of the sum of scores by each dimension between before-treatment and end-of-treatment. Additionally, subgroup analyses of the symptom score and the score of quality of life were conducted according to age, infection of *H pylori* and sex.

RESULTS

Patient characteristics

A total number of 272 patients (133 patients in ecabet group, 139 patients in cimetidine group) with functional dyspepsia were enrolled. In both groups, there was no significant difference in demographic characteristics including age, sex, weight, height, rate of smokers, and rate of alcohol intake (Table 1). Thirty-six patients (18 patients in ecabet group, 18 patients in cimetidine group)

Table 1 Demographic characteristics of the enrolled patients

Group	Ecabet group (n = 133)	Cimetidine group (n = 139)	P
Gender (male:female)	47:86	47:92	NS
Age (yr)	45.0±12.9	47.5±12.5	NS
Weight (kg)	59.6±10.0	58.5±9.0	NS
Height (cm)	162.7±8.0	161.8±7.7	NS
Smoking (%)	23 (17.3)	16 (11.5)	NS
Alcohol intake (%)	47 (35.3)	38 (27.3)	NS

Table 2 Prevalence of *Helicobacter pylori* infection

Group	Positive (%)	Negative (%)	Undetermined (%)
Ecabet group (n=120)	44 (36.7)	45 (37.5)	31 (25.9)
Cimetidine group (n=128)	40 (31.3)	60 (46.9)	28 (21.9)

Pearson's chi-square test; P=NS.

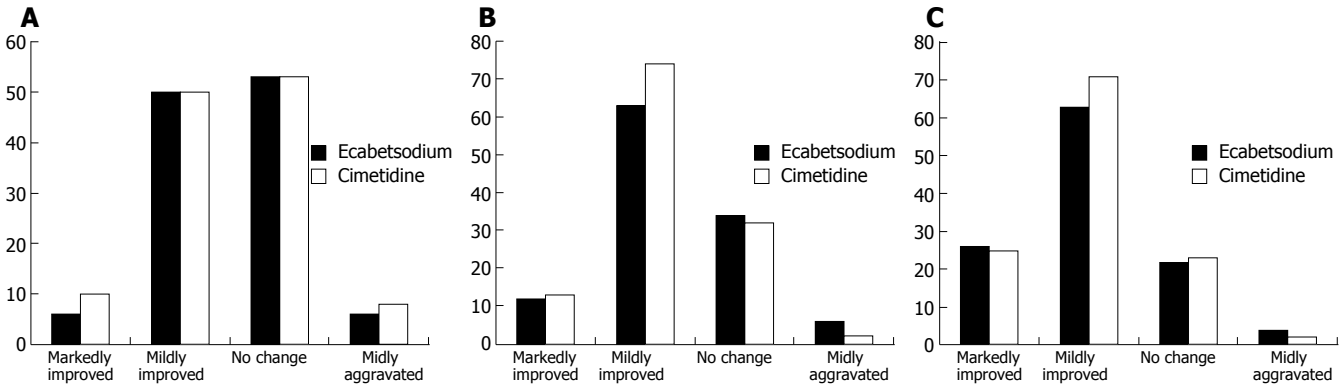


Figure 1 Comparison of the rate of the symptomatic improvement in both groups at 3 d (**A**), 2 wk (**B**) and 4 wk (**C**) of medication.

Table 3 Global assessment of symptomatic improvement after 4 wk of medication

Group	Ecabet group (n = 115) (%)	Cimetidine group (n = 121) (%)
Improved	89 (77.4)	96 (79.3)
Not improved	26 (22.6)	25 (20.7)

Pearson's chi-square test: P=0.71.

of them were dropped out. A total of 236 patients (115 patients in ecabet group, 121 patients in cimetidine group) were included in the per-protocol analysis. There was no significant difference between two groups in medication compliance 2 and 4 wk after administration. There was no significant difference between treatment groups with the respect to the proportion of patients with *H pylori* infection (Table 2).

Improvement of symptoms

For the measurement of the overall rate of improvement, a question for global symptomatic assessment was used. The result of evaluation of symptoms at baseline, 3 d, 14 d and 28 d after treatment demonstrated no significant difference between the groups (Figures 1A-C). However, both groups showed significant improvement of symptoms at 2 and 4 wk compared to the baseline (Figures 1B, IC).

When significant improvement and somewhat improvement were considered as improved, and no change, somewhat worsen, and severely worsen as not improved, the rate of improvement at 4 wk was not different between ecabet group and cimetidine group (Table 3) (P=0.53). The sum of 12 symptom scores of both groups was not different at 3 d, 2 wk and 4 wk (P=0.84).

Quality of life

The parameters of quality of life were categorized into eating status, liveliness status, psychological status, and role-functioning status. In both groups, the sum of quality of life scores in each category did not change significantly after treatment (Table 4).

Change of laboratory results

After treatment, there was no significant change in laboratory parameters such as WBC count per mm^3, ESR, and CRP in both groups.

Subgroups analysis: H pylori status, age and sex

The patients were grouped by serologic result with *H pylori*-positive group and -negative group. With the respect to the global improvement of symptoms, there was no significant difference between two groups regardless of *H pylori* infection (Table 5). In the subgroups by the age, categorized with less than 50 years old and equal/over 50 years old groups, there was no difference in the global symptom improvement in both groups (Table 6). In the subgroups by the gender, there was also no difference in the global symptom improvement in both groups (Table 7).

Adverse effects

There was no severe adverse event during the study period. Three patients among 133 patients in the ecabet group reported mild adverse events, such as diarrhea, rash

Table 4 Comparison of quality of life scores before and after treatment (mean ± SD)

Status	Before treatment			After treatment		
	Ecabet group (*n* = 115)	Cimetidine group (*n* = 121)	*P*	Ecabet group (*n* = 115)	Cimetidine group (*n* = 121)	*P*
Eating status	15.0±4.6	14.8±4.1	NS	15.6±4.0	15.9±3.5	NS
Liveliness status	10.4±4.1	10.0±4.0	NS	11.6±3.6	11.3±3.5	NS
Psychological status	18.5±5.0	18.6±4.5	NS	20.3±4.4	20.4±4.1	NS
Role-functioning status	20.5±4.2	20.6±4.2	NS	21.4±3.9	21.0±4.3	NS

Table 5 Comparison of global symptomatic improvement by *H pylori* status

H pylori infection	Gastrex group		Cimetidine group	
	Improved (%)	Not improved (%)	Improved (%)	Not improved (%)
Negative	28 (84.85)	5 (15.15)	27 (84.38)	5 (15.63)
Positive	28 (75.68)	9 (24.32)	24 (72.73)	9 (27.27)

Chi square test, $P = 0.89$.

Table 6 Comparison of global symptomatic improvement by age

Age	Gastrex group		Cimetidine group	
	Improeed (%)	Not improved (%)	Improved (%)	Not improved (%)
Less than fifty	55 (61.8)	14 (53.85)	61 (63.54)	12 (48.0)
fifty and over	34 (38.2)	12 (46.15)	35 (36.46)	13 (52.0)

Chi square test, $P = 0.63$.

Table 7 Comparison of global symptomatic improvement by sex

Sex	Gastrex group		Cimetidine group	
	Improved (%)	Not improved (%)	Improved (%)	Not improved (%)
Men	27 (71.05)	11 (28.94)	34 (79.07)	9 (20.93)
Women	63 (80.76)	15 (19.23)	62 (79.48)	16 (20.51)

Chi square test, $P = 0.79$.

and abdominal pain. However, the events were treated with simple symptomatic treatments. Two patients among 139 patients in the cimetidine group complained of mild diarrhea. In the chemistry at 4 wk, mild transient elevation of transaminases occurred in 1 case in the ecabet group and 1 case in the cimetidine group, respectively.

DISCUSSION

Functional dyspepsia is a symptom complex characterized by postprandial upper abdominal discomfort or pain, early satiety, nausea, vomiting, abdominal distention, bloating, and anorexia in the absence of organic disease. In this study, we used the Rome-II criteria for the exact identification of patients with functional dyspepsia[1].

The major pathophysiological mechanisms responsible for functional dyspepsia include psychosocial factors and alterations in motility and visceral perception. Approximately 50% of patients with functional dyspepsia have motor disorders, such as impaired fundic relaxation, antral dilatation and/or hypomotility, gastroparesis. It is also observed that symptoms of functional dyspepsia are related to or worsen by several psychological factors, such as negative feelings as anxiety and/or depression, abnormal stress response, dependence personality, change of coping strategy, *etc*[2-4]. The important mental element related with patients with functional dyspepsia is stress of daily life. At present, administration of antidepressants has been found to be useful for only a few patients, control of psycho-sociable element is not assessed yet[2].

Ecabet sodium (ES) is a newly developed anti-inflammatory/anti-ulcer agent and belongs to the category of gastroprotective agents[14-16]. Proposed mechanisms of action include improved blood flow in the gastric mucosa, increased gastric mucin, increased gastric mucosal PG E2, and decreased activity of pepsin[15,19]. It was reported that ES increases the eradication rate of Helicobacter pylori in dual therapy with lansoprazole and amoxicillin[18]. In patients with gastric ulcer treated with cimetidine, additional use of ES significantly accelerates rates of ulcer healing and symptomatic relief[19]. Maintenance therapy with a combination of ranitidine and ES prevents ulcer relapse in Helicobacter pylori-positive patients[20]. Interestingly, ES enemas have been proved to be a safe and potentially useful adjuvant therapy in patients with mildly to moderately active ulcerative proctosigmoiditis[21].

There are only a few clinical studies examining the effect of mucosal protective agents in patients with functional dyspepsia. A double-blind placebo-controlled multicenter study examining the effect of rabamipide in patients with functional dyspepsia reported that there is no significant improvement of individual symptom scores in rebamipide group compared to placebo group[23]. However, the efficacy of ES for patients with functional dyspepsis has never been reported in the literature. In this study examining the efficacy of ES in patients with functional dyspepsia, ES showed equivalent outcomes in most clinical indexes compared to a representative H_2 receptor antagonist (cimetidine). Rates of improvement of global symptoms were not different at 3 d, 14 d, and 28 d after starting treatment (Table 3). At the end of the 4-wk treatment, 77.4% of patients in ecabet group and 79.3% of patients in the cimetidine group reported improvement of global symptoms ($P > 0.05$). In the analysis of 12 individual symptoms, there was no significant difference

in both groups. In the subgroup analysis by age, gender and *H pylori* infection status, we could not find any factors affecting the rate of improvement.

Health-related quality of life is becoming increasingly recognized as an important outcome for patients with chronic diseases[24]. Patients with functional dyspepsia can experience significant levels of abdominal pain that interrupt daily activities, so quality of life is impaired[25]. In patients with functional dyspepsia, therefore, the impact of treatment on the quality of life can be a good measure of treatment outcome. For the evaluation of the quality of life, we used the IDS-K questionnaire, which was recently developed and validated in Korea[22]. Despite improvement of dyspeptic symptoms during the treatment period, we could not find any significant change in any dimension of our questionnaire before and after the 4-wk treatment (Table 4). One possible reason for this discrepancy may be that the duration of 4-wk treatment may be too short to document a significant change in quality of life. Longer-term treatment may be necessary for the exact evaluation of the impact of treatment on the quality of life in dyspeptic patients.

The major limitation of the present study is the lack of treatment arm using placebo. In this study, we could see a significant improvement of symptoms in both ecabet group and cimetidine group. The reported response rates of cimetidine for patients with non-ulcer dyspepsia are quite variable. Nyren *et al*[26] reported that the mean reduction in pain intensity after three weeks is less than 30% in both placebo and cimetidine group. In a meta-analysis of placebo-controlled studies for non-ulcer dyspepsia, the mixed response rate for cimetidine treatment is 67.3% (272/404)[27]. In the present study, the response rate of ecabet group was 77.4%, and that of cimetidine group was 79.3% (Table 3). The rate of improvement in this study seemed to be a little bit higher than that in the previous studies. In our opinion, differences in the patient population and the definition of improvement may be possible explanations. In the present study, there was no arm for the placebo, so we could not exclude the possibility that the outcome of this study might be a placebo effect. In the previously mentioned meta-analysis, however, the mixed response rate for placebo is 49.1% (183/372)[25]. In order to determine the efficacy of ES for functional dyspepsia more exactly, long-term randomized clinical trial with placebo arm is mandatory.

In conclusion, 4-wk treatment with ES demonstrates equivalent symptom improvement compared to a representative H_2-receptor antagonist (cimetidine) in patients with functional dyspepsia.

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CLINICAL RESEARCH

Expression of angiostatin cDNA in human gallbladder carcinoma cell line GBC-SD and its effect on endothelial proliferation and growth

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Abstract

AIM: To explore the influence of angiostatin up-regulation on the biologic behavior of gallbladder carcinoma cells *in vitro* and *in vitro*, and the potential value of angiostatin gene therapy for gallbladder carcinoma.

METHODS: A eukaryotic expression vector of pcDNA3.1(+) containing murine angiostatin was constructed and identified by restriction endonuclease digestion and sequencing. The recombinant vector pcDNA3.1-angiostatin was transfected into human gallbladder carcinoma cell line GBC-SD with Lipofectamine 2000, and paralleled with the vector and mock control. The resistant clone was screened by G418 filtration. Angiostatin transcription and protein expression were examined by RT-PCR, immunofluorescence and Western-blot. The supernatant was collected to treat endothelial cells. Cell proliferation and growth *in vitro* were observed under microscope.

RESULTS: Murine angiostatin cDNA was successfully cloned into the eukaryotic expression vector pcDNA3.1 (+). After 14 d of transfection and selection with G418, macroscopic resistant cell cloning was formed in the experimental group transfected with pcDNA 3.1(+)-angiostatin and vector control. But untreated cells died in the mock control. Angiostatin was detected by RT-PCR and protein expression was detected in the experimental group by immunofluorescence and Western-blot. Cell proliferation and growth *in vitro* in the three groups were observed respectively under microscope. No significant difference was observed in the growth speed of GBC-SD cells between groups that were transfected with and without angiostatin. After treatment with supernatant, significant differences were observed in endothelial cell (ECV-304) growth *in vitro*. The cell proliferation and growth were inhibited.

CONCLUSION: Angiostatin does not directly inhibit human gallbladder carcinoma cell proliferation and growth *in vitro*, but the secretion of angiostatin inhabits endothelial cell proliferation and growth.

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Key words: Angiostatin; Gallbladder carcinoma; Endothelial cell

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INTRODUCTION

Gallbladder carcinoma is the most common malignant tumor of the biliary tract and a particularly high incidence is observed in Chile, Japan, and northern India. After common treatment, the prognosis of gallbladder carcinoma patients is poor[1].

A large body of work by a number of laboratories in the past 3 decades has provided both direct and indirect evidence that tumor growth and metastasis are accompanied with the growth of new blood vessels[2].

As previously reported, angiostatin is a potent antiangiogenic endogenous protein[3]. In the present study, we constructed a mammalian expression vector that was cloned as an angiostatin gene. Following stable transfection of this vector into human gallbladder carcinoma cell line GBC-SD, we observed the expression of angiostatin and its antiangiogenic effect by evaluating the influence of cultured human umbilical vein endothelial cells (ECV-304).

MATERIALS AND METHODS

Cell lines and reagents

The human gallbladder carcinoma cell line GBC-SD and the endothelial cell line ECV-304 were purchased from

China Center for Type Culture Collection. EcoRI, XbaI, PmeI, HindIII, XbaI and PvuIwere purchased from Bioson Corporation. Lipofectamine™ 2000 and G418 were purchased from Gibco Company. Recombinant eukaryotic expression vector pcDNA3.1(+)-angiostatin and rabbit anti-HAtag multiclonl antibody were presented by Dr. Jicheng Zhang. Trizol reagent, reverse transcriptase, and Taq DNA polymerase were purchased from Life Technologies, Inc. FITC was purchased from Boster Biological Technology Co.

Cell culture

Gallbladder carcinoma cells and endothelial cells were cultured in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), penicillin (100 units/mL) and streptomycin (100 mg/mL) in a humidified atmosphere containing 5% CO_2 at 37 ℃.

Recombinant eukaryotic expression vector pcDNA3.1(+)-angiostatin construction

Mouse angiostatin cDNA fragment encoding for the NH2-terminal secretory signal sequence(SS) and kringle1-4(K1-4) regions of mouse plasminogen, fused with an antigenic epitope tag HA(HA tag) to the COOH terminus of kringle 4, was inserted into enkaryotic expression vector pcDNA3.1(+). The structure of the recombinant vector pcDNA3.1(+)-angiostatin was confirmed by restriction endonuclease digestion and sequencing. Recombinant clones were identified by HindIII, XbaI and PmeI digestion. Positive clones were further confirmed by sequencing.

Transfection of pcDNA3.1-angiostatin into GBC-SD

Gallbladder carcinoma cells (GBC-SD) in logarithmic growth phase were plated on 24-well plates at 2×10^5 cells/well, and approximately 80% confluence was obtained after overnight incubation. Cultured GBC-SD cells were divided into three groups: transfected with recombinant pcDNA3.1-angiostatin (group A), pcDNA3.1 (+) vector (group B) and without pcDNA3.1 (+) vector(group C). Transfection was performed according to the instructions of Lipofectamine TM2000 reagent kit (Gibco). The cells were then cultured in complete medium containing G418 (50 mg/L) for 14 d. G418-resistant pooled cells were subjected to further studies. Then the isolated resistant cell clones were selected and amplified. Cell growth curve was also plotted.

Analysis of angiostatin transcript by RT-PCR

Total RNA was isolated from GBC-SD cells using Trizol reagent. Reverse transcription was performed with 1 ug of total RNA in a total volume of 20 μL containing reverse transcriptase. The PCR reagents, including 0.5 units of Taq DNA polymerase, were added to a final volume of 25 μL. A 35-cycle amplification profile consisted of denaturation at 94 ℃ for 1 min, annealing at 56 ℃ for 1 min, and extension at 72 ℃ for 1 min. Primers used in PCR were designed according to the reported angiostatin cDNA sequence. The primer sequences were as follows: 5'end: 5' -CCCAACATGGACCATAAGGAAGT-3' and 3'end: 5' -TGTGGGCAATTCCACAACACTC-3'. Human-actin primers were used as positive controls. Negative controls without RNA and reverse transcriptase were also assessed. The PCR products were identified by 1% agarose gel electrophoresis.

Analysis of angiostatin protein by Western blot and immunofluorescence cytochemistry

GBC-SD cells with pcDNA3.1(+)angiostatin in logarithmic growth phase were implanted into 6-well plates. After 24 h, the glass flake was taken out and imunofluorescence cytochemistry was carried out. For immunofluorescence, cells on glass flake were fixed for 15min in 3.7% formaldehyde in PBS containing 1mM EGTA at room temperature. After fixation, the cells were made permeable by incubating with 0.2% Triton X-100 in PBS for 15 min, and then washed with PBS. After being blocked with PBS including 1% BSA, antibodies were applied and incubated for 1h at 37 ℃. The cells were then stained with secondary antibodies. After being washed with PBS, the expression was detected under fluorescent microscope.

The cells in the three groups were cultured in conditioned media for 5 d. Cell supernatant was mixed with lysine-sepharose and incubated at 4 ℃ overnight. The resin was washed with Tris-HCl (pH8.0) and protein was eluted. Protein concentration was determined by bicinchoninic acid assay (BCA) with bovine serum albumin as standard. Equal aliquots (40 μg) of protein from cell supernatants were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred onto PVDF membranes (mMillipore) using the transfer buffer for 2 h. The membranes were then blocked for 1.5 h using 5% nonfat dried milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T), washed with PBS-T, and incubated at 4 ℃ overnight in the presence of anti-rabbit HA-tagged antibody.The membranes were washed with PBS-T and incubated with secondary peroxidase-conjugated anti-rabbit immunoglobulin G for 1 h. Following washing with PBS-T, immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham Life Science).

Viable cell number counting and growth curve

In logarithmic growth phase of the three groups of gallbladder carcinoma cells (GBC-SD), the supernatant was collected. Endothelial cells (ECV-304) were plated onto 24-well plates at 1×10^4 cells/well and ECV-304 cells were treated with supernatant separately for 7 d (supernatant/medium=1/9). Viable cells were counted under microscope everyday and growth curves were plotted.

Statistical analysis

Data were analyzed by statistical software of SPSS 10.0. Differences between groups were examined by Student's *t* test. $P<0.01$ was considered statistically significant.

RESULTS

Identification of recombinant plasmid pcDNA3.1(+)-angiostatin

The recombinant plasmid was released by restrictive digestion with HindIII, XbaI and PmeI. As shown in electro-

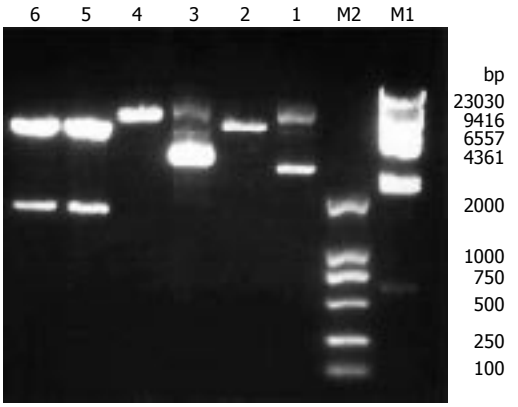


Figure 1 Identification of angiostatin cDNA insertion in pcDNA3.1(+)-angiostatin. M1: λDNA/Hind Ⅲ marker; M2: DNA marker DL2000; lane 1: pcDNA3.1(+)plasmid; lane 2: pcDNA3.1(+)/*Eco*RⅠ; lane 3: pcDNA3.1(+)-angiostatin; lane 4: pcDNA3.1(+)-angiostatin/XbaⅠ; lane 5: pcDNA3.1(+)-angiostatin/*Pme*Ⅰ; lane 6: pcDNA3.1(+)-angiostatin/ *Hind* Ⅲ +*Xba*Ⅰ.

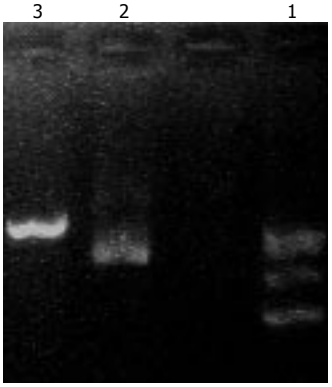


Figure 2 pcDNA3.1(+)-angiostatin linearization. Lane 1: λDNA/*Hind* Ⅲ marker; lane 2: pcDNA3.1(+)-angiostatin; lane 3: pcDNA3.1(+)-angiostatin /*Pvu*Ⅰ.

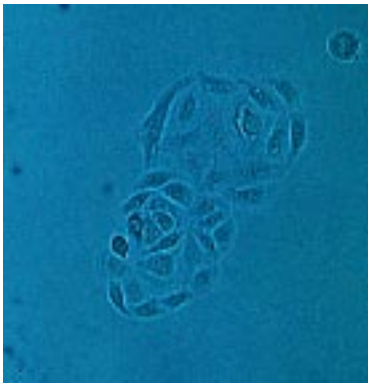


Figure 3 Gallbladder carcinoma cell clones transfected with pcDNA3.1(+)-angiostatin.× 400

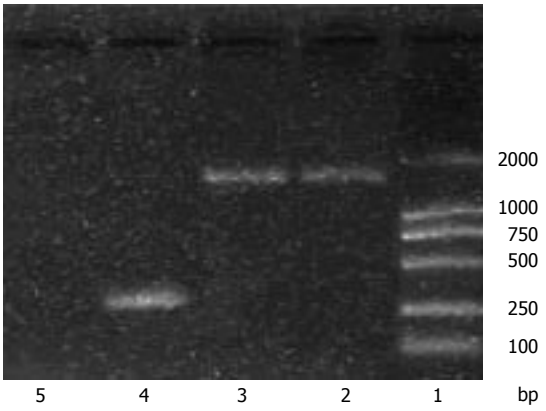


Figure 4 Analysis of angiostatin transcript by RT-PCR. Lane1: DNA marker DL2000; lanes 2, 3: angiostatin; lane 4: positive control; lane 5: nagetive control.

phoresis, the linear recombinant plasmid was about 7.0kb, being a fragment of 1.4kb, suggesting that angiostatin fragment was inserted into the pcDNA3.1 (+) vector, named recombinant plasmid pcDNA3.1- angio (Figure1). The sequence obtained was the same as the reported sequence of angiostatin cDNA, indicating that the murine gene was successfully cloned into the eukaryotic expression vector pcDNA3.1 (+).The recombinant plasmid was linearized by *pvu* I(Figure 2).

Vector-mediated expression of angiostatin in vitro

Gallbladder carcinoma cells (GBC-SD) transfected with the corresponding vectors were selected by G418 for 14 d. The transfected pcDNA3.1-angiostatin and vector control groups formed macroscopic cell clones (Figure 3), but the mock group of cells was completely dead after 8 d of selection.

Angiostatin expression in transfected cells

GBC-SD cells transfected with pcDNA3.1- angiostatin were prepared. Total RNA was extracted and used as the template. A band was detected at 1.4 kb with PCR using angiostatin primers, indicating the presence of angiostatin cDNA in the GBC-SD cancer cells (Figure 4).

Angiostatin protein expression in vitro

Immunofluorescence showed signals of angiostatin in the experimental group of cell clones genetically engineered but not in the controls (Figure 5A). Supernatants of cultured cells in three groups were collected and analyzed by Western blotting. A band in a molecular mass of 58 000 kb was detected with rabbit anti-HA-tagged antibody from the cells transfected with pcDNA3.1-angiostatin, but no band was detected in mock control group or vector control group (Figure 5B).

Biological activity of angiostatin protein expression in vitro

To detect the biological activity of encoded angiostatin *in vitro*, tumor cells transfected with or without the corresponding vectors were cultured for 7 d to plot cell growth curve (Figure 6A). Under microscope, no obvious difference was observed in the cell morphology of the three groups of cells. Cell growth curves indicated no change in cell growth speed and doubling time in the three groups. These results indicate that up-regulated angiostatin expression could neither directly inhibit cell growth and proliferation nor affect cell cycle *in vitro*. But the biological activity of cells treated with supernatant of GBC-SD/ pcDNA3.1(+)-angiostatin and cell growth were obviously inhabited. The cell growth curve of the vector and mock control groups was similar (Figure 6B). These results indicate that angiostatin expression could directly inhibit endothelial cell growth and proliferation *in vitro*.

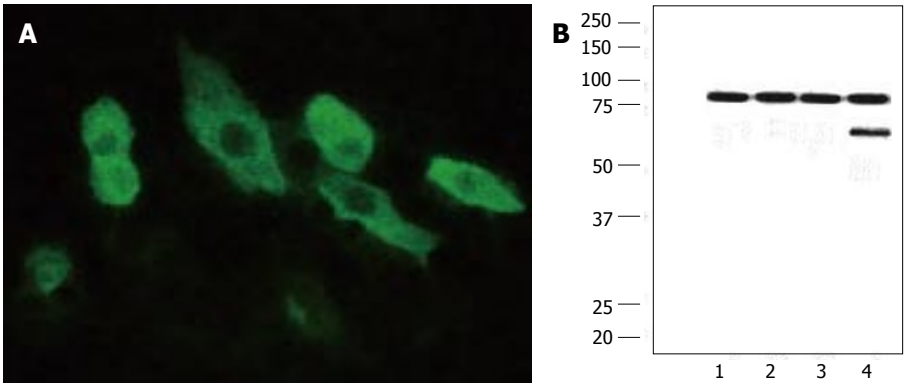


Figure 5 Angiostatin protein expression by immunofluorescence(**A**) and Western blot analysis(**B**). M:Marker; lane 1: PBS control; lanes 2,3: GBC-SDpcDNA3.1(+); lane 4: GBC-SDpcDNA(3.1)angiostatin.

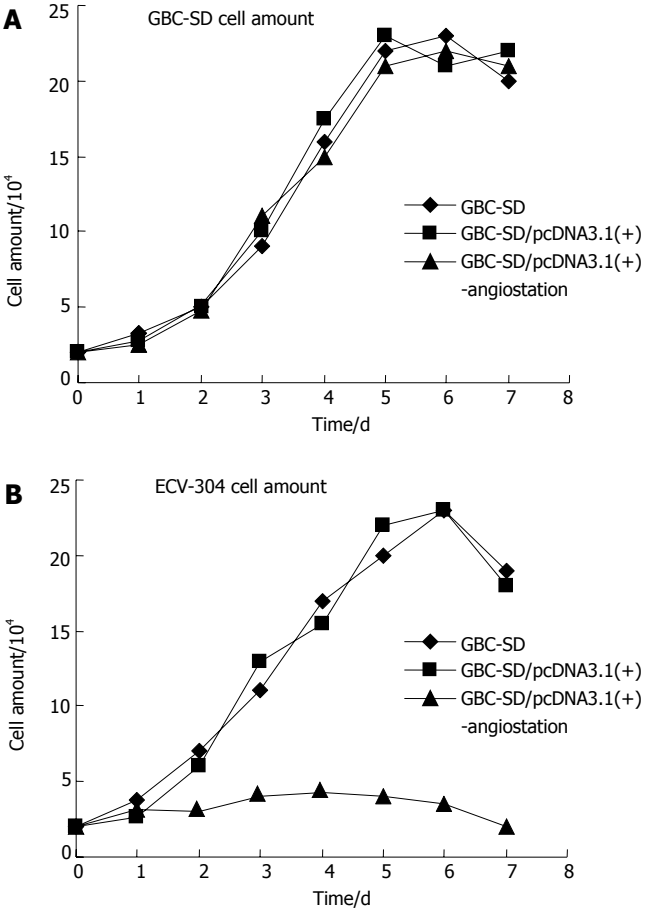


Figure 6 Cell growth curves of GBC-SD(**A**) and ECV-304(**B**).

DISCUSSION

Angiogenesis refers to the formation of new blood vessels. Since Dr. Folkman[15] raised the hypothesis of tumor angiogenic dependence, experimental evidence has validated that tumor growth companies new blood vessel growth. At the prevascular stage, tumor is unable to grow to a size beyond 2-3 mm^3 and remains in its dormant state. However, once the angiogenic phenotype of tumor is switched on, tumor growth rate changes from linear to exponential[4-6]. Infiltration of new blood vessels in tumors not only supplies nutrients and oxygen for tumor cells, but also removes the waste products produced by tumor cells. In addition, endothelial cells can communicate directly with tumor cells by producing tumor growth promoting factors.

Angiogenesis begins when a fibrin clot forms on the adventitial surface of an existing blood vessel[7], which is followed by sprouting of new capillaries. The initial phase begins with increased vascular permeability and local degradation of the vessel wall. Endothelial cells enter the tumor stroma and proliferate. At this time, the cells may be most vulnerable to agents that interfere with their proliferation, since they lack protection from other cell types[8]. The next step in vessel formation is recruitment of pericytes, followed by smooth muscle cells.

It appears that a switch to the angiogenic phenotype requires a local change in the balance between angiogenic factors and inhibitors[9]. Among the angiogenic factors, fibroblast growth factor (FGF)[10] and vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF)[11] are most commonly expressed in tumors. Others are inhibitors such as angiostatin[12], thrombospondin-1[13], 16-kd prolactin fragment[14], interferon-α and β[15], and endostatin[16]. Because cancer cannot grow or spread without the formation of new blood vessels[17], scientists are trying to find ways to stop angiogenesis.

Tumor angiogenesis is often the consequence of an angiogenic imbalance in which pro-angiogenic factors predominate over anti-angiogenic factors[5]. Furthermore, angiogenesis is essential for growth and metastasis of most solid cancers[18]. Gallbladder carcinoma is not a grossly vascular tumor, but is related to angiogenesis[19]. Antiangiogenic treatment may be necessary and potential for gallbladder carcinoma.

Angiostatin is an internal fragment of plasminogen and may contain either the first three (K1-3) or four (K1-4) kringle domains[20]. A similar activity has been reported for kringle 5 (K-5) of plasminogen[21]. Three receptors of angiostatin on endothelial cells have been reported to date. Adenosine triphosphate (ATP) synthase is one of the receptors. The presence of this typically mitochondrial enzyme on the endothelial cell surface is somewhat surprising[22]. Two other potential target receptors of angiostatin are angiomotin[23] and integrin alpha(v)beta(3)[24]. These receptors on the surface of endothelial cells may be located at sites so proximal to one another that angiostatin may be able to interact with more than one simultaneously[25]. Alternatively, multiple targets could be implicated in binding to different kringles of angiostatin as a function of receptor and peptide presentation[26].

Although angiostatin is a potent inhibitor of angiogenesis and tumor growth[27], the need for high doses, repeated injections and long-term administration of this protein

have made it less attractive for clinical trials[28]. In order to develop alternative strategies for therapy, we have investigated the possibility of angiostatin gene therapy. When mouse angiostatin cDNA was transfected into human gallbladder carcinoma cell line GBC-SD and endothelial cell line (ECV-304), angiostatin cDNA was present in cancer cells, and a secreted form of angiostatin was established by immunofluorescence and Western-blot. Although angiostatin had no direct effect on gallbladder carcinoma cell growth *in vitro*, the supernatant of ECV-304 cells inhibited the growth of endothelial cells *in vitro*. Xu *et al*[29] engineered a recombinant adeno-associated virus (AAV) vector encoding mouse angiostatin and found that it suppresses metastatic liver cancer in mice. In our study, similar results were achieved using Lipofectamine 2000 for transfection. These results support that angiostatin-gene therapy is a potential strategy in the treatment of gallbladder carcinoma.

In conclusion, angiostatin has no direct effect on gallbladder carcinoma cell growth *in vitro*, but inhibits the growth of ECV-304 cells *in vitro*.

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RAPID COMMUNICATION

Effect of somatostatin analogue octreotide injected into the third cerebral ventricle on pentagastrin-induced gastric acid secretion in rats

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Abstract

AIM: To investigate the effect of long-lasting somatostatin analogue octreotide (Oct) injected into the third cerebral ventricle (TCV) on gastric acid secretion in rats.

METHODS: TCVs were cannulated in male Wistar rats anesthetized with sodium pentobarbital. One week later acute gastric lumen perfusion was carried out and gastric acid was continuously washed with 37℃ saline by a perfusion pump. Gastric perfusion samples were collected every 10 min and titrated by 0.01 moL/L NaOH to neutral. On the basis of subcutaneous (sc) injection of pentagastrin (G-5, 160 μg/kg), Oct (0.025 μg, 0.05 μg, 0.1 μg, $n = 12$ in each group) or vehicle (pyrogen-free physiological saline, $n = 10$) was injected into the TCV. Before and after the TCV injection, 1 h total acid output (TAO) was determined and experimental data were expressed in change rate (%) of TAO.

RESULTS: Oct (0.025, 0.05 and 0.1 μg) injected into the TCV resulted in change rate of 1.56% ($P > 0.05$), 20.21% ($P < 0.01$) and 37.82% of TAO ($P < 0.001$), respectively. Moreover, comparison in change rate of TAO among these 3 doses showed $P < 0.05$ between 0.025μg and 0.05 μg, $P < 0.01$ between 0.025 μg and 0.1μg, and $P < 0.05$ between 0.05μg and 0.1 μg. However, sc injection of 0.05 μg Oct had no effect on G-5 stimulated gastric acid secretion.

CONCLUSION: Octreotide injected into the third cerebral ventricle inhibits gastrin-induced gastric acid secretion in a dose-dependent manner.

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Key words: Octreotide; Somatostatin analogue; Third cerebal ventricle; Gastric acid; Gastrin

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INTRODUCTION

Somatostatin, a brain-gut peptide, distributes widely in brain tissue, gastrointestinal tract, pancreatic islets, etc and plays a diversified role in biological activities[1]. Due to its short half life, its application in basic research and clinical work is limited. Octreotide (Oct) is a long-lasting somatostatin analogue with a plasma half life of 45 min[2]. Clinically it is used in the treatment of acromegaly[3], gastrointestinal hemorrhage[4] as well as in the diagnosis and management of some tumors[5]. In the aspect of basic research on gastric acid secretion there have been few reports concerning the central administration of somatostatin and its analogues. Some results are contradictory. Such discrepancy may result from differences in animal species, experimental models and methods, types, doses and injected route of the drugs used. Of note, previous studies[6,7] strongly suggested that the site of injection of Oct into the brain could largely influence its effect on gastric acid secretion. Up to now there is no report regarding the effect of somatostatin and its analogues into the third cerebral ventricle (TCV). In the present study Oct was injected into the TCV of the rats via a chronically implanted cannula and its effect on gastrin-induced gastric acid secretion was observed in the process of acute gastric lumen perfusion.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 180-280 g were used. The

Table 1 Effect of sc injection of G-5 or TCV injection of saline on gastric acid secretion (n = 10, mean ± SE)

Index	sc G-5		TCV saline	
	1st time	2nd time	before	after
TAO (μmoL/h)	23.93 ± 3.83	26.66 ± 5.15	18.24 ± 1.72	18.86 ± 1.38
Change rate of TAO (%)	8.08 ± 5.46		5.90 ± 5.47	

Table 2 Effect of TCV-injected Oct on G-5 stimulated gastric acid secretion (mean ± SE)

Group	n	TCV injection	Change rate of TAO (%)	P
1	10	Saline 2.5 μL	5.90 ± 5.47	
2	12	Oct 0.025 μg/2.5μL	1.56 ± 6.63	> 0.05
3	12	Oct 0.05 μg/2.5μL	20.21 ± 6.49[a]	< 0.01
4	12	Oct 0.1 μg/2.5μL	37.82 ± 3.97	< 0.001

[a]P < 0.05 *vs* group 2 or group 4.

animals were deprived of food for 24 h, but allowed free access to water prior to anesthesia.

Animal models

The rats were anesthetized with a single intraperitoneal injection of sodium pentobarbital (50 mg/kg). Intra-TCV implantation and acute gastric lumen perfusion were carried out as described previously[8]. Gastric perfusion samples were collected every 10 min and titrated by 0.01 moL/L NaOH to neuter (using phenol red as a volume marker). The acid output per 10 min was calculated as previously described[9]. The anus temperature of rats was kept at 37℃ by electric light during the experiment. Sufficient pentobarbital was given sc before G-5 was injected. By referring to the previous report, G-5 (160 μg/kg) was chosen to achieve the maximal acid output[10]. After basal gastric acid secretion was kept stable for 30 min, G-5 (Sigma Company, USA) was injected sc, followed by collection of gastric perfusion samples at 10 min intervals for 1 h. Gastric acid secretion increased 10 min after the injection, reached its peak at 20 min, then declined gradually to the basal level 1 h after the injection. After the gastric acid secretion was kept stable at the original basal level for 30 min, the same dose of G-5 as the first time was given sc and gastric perfusion samples were collected for another 60 min. The experimental drugs were administered into TCV 10 min before the second injection of G-5. In brief, 2.5 μL of pyrogen-free physiological saline or various doses of Oct (Sandoetatin Norvartis, Basel, Switzerland) were given by a syringe pump (with the constant speed of 2.5 μL/2 min) in a silicon tube, which was equally long and connected with the implanted cannula. In order to prove the cannula was in TCV[9], 5 μL gentian violet was injected via the implanted cannula at the end of the experiment, and the rat was immediately killed. The brain tissue was removed and cut to see whether TCV was stained by gentian violet. TCV staining signified that the cannula was correctly placed in TCV and the injected drug successfully reached TCV. Therefore, the result was accepted only when TCV was stained. In this experiment, only data arising from the two rats in saline-injected group were excluded because their TCVs were not shown on gentian violet staining.

Statistical analysis

Experimental data was expressed in percent age of TAO change, which was calculated as follows: change of TAO (%) = (E2-E1)/E1×100%, in which E1 and E2 represent 1 h TAO (μmol/L) after the first and the second sc injections of G-5, respectively. The data were expressed as mean ± SE. Significance was assessed by unpaired t test for comparison between two groups while paired t test for comparison between pre-treatment and post-treatment in each group.

RESULTS

Effect of sc injection of G-5 or TCV injection of saline on gastric acid secretion

The effect of sc injection of G-5 (160 μg/kg) two times on basal gastric acid secretion was observed in the same rat without cannulization in TCV (Table 1). The result showed that there was no significant difference in G-5 stimulated TAO between the first and second injections (P > 0.05). The effect of pyrogen-free physiological saline (2.5 μL) given into TCV on G-5 induced gastric acid secretion in the rat cannulated in TCV, revealed that TAO was almost identical between pre- and post-treatment of TCV-injected saline (P > 0.05, Table 2). Moreover, comparison in change of rate of TAO between these two groups demonstrated an insignificant difference (P > 0.05). This strongly suggested that the experimental animal model was so stable that it could be employed in study of the effect of TCV-administered drugs on gastric acid secretion in rats.

Effect of sc injection of Oct on G-5 induced gastric acid secretion

In 10 rats without cannulation in TCV, each received two injections of sc G-5. Saline (0.20 mL) or Oct (0.05 μg/0.20 mL) was given sc 10 min before the first or the second G-5 stimulation, respectively. TAO was 34.41 ± 3.75 μmol/h after saline administration and 33.49 ± 3.98 μmoL/h after Oct treatment, which had no significant difference between them (P > 0.05).

DISCUSSION

Injection of brain-gut peptides (*eg*, somatostatin, neuropeptide Y, corticotropin-releasing factor, bombesin) into the central nervous system can influence gastric acid secretion[11-13]. As a long-lasting somatostatin analogue Oct, intracisternal injection (ic) induces a dose-related (100-300 ng) and long-lasting stimulation of gastric acid output in pylorus-ligated conscious rats[6]. Four years later, the same researchers reported that Oct injected into the different regions of the brain displayed its effect on gastric acid secretion[7]. Interestingly, they found that Oct (7, 15, 30 and 60 ng) injected into the dorsal vagal

complex (DVC), increases pentagastrin-stimulated gastric acid secretion of urethane-anesthetized rats in a dose-dependent manner. Whereas, Oct injected into the lateral ventricle (100, 200, 300 ng), paraventricular nuclei (PVN) or lateral hypothalamus (LH) (7.5, 15, or 30 ng) also inhibits pentagastrin-stimulated gastric acid secretion in a dose-dependent manner. Oct (30 ng) injected into the area surrounding the PVN or LH does not modify the acid secretion response to pentagastrin. These results strongly suggest that the injection sites of Oct in the central nervous system influence their effect on gastric acid secretion. The current study, for the first time, explored the effect of intra-TCV injection of Oct on gastric acid secretion in rats and showed that Oct (0.025, 0.05, 0.1 μg) injected into TCV inhibited pentagastrin-induced gastric acid secretion of pentobarbital-anesthetized rats with chronically implanted cannula in a dose-dependent manner.

Without no doubt, somatostatin and its analogues peripherally inhibit gastric acid secretion[14]. There is evidence that somatostatin-14 injected into the lateral cerebral ventricles at 3-6 nmoL/rat induces massive hypersomatostatinemia resulting from leakage into the peripheral circulation of rats[15]. Our present study revealed that gastric acid output was suppressed by TCV-administrated Oct through a chronically implanted cannula. Whether Oct injected into TCV is absorbed into blood via the capillaries of subarachnoid space, choroid plexus and cerebral parenchyma to function peripherally is unknown, but Oct is unlikely to enter blood directly due to acute damage to the blood vessel. However, sc injected Oct at the dose of 0.05 μg in our study did not have any effect on G-5 induced gastric acid secretion, thus excluding the peripheral action of Oct. Moreover, it was reported that somatostatin could not pass through the intact blood brain barrier in humans[16].

Hypothalamus plays an important role in the central regulation of gastric acid secretion[17]. Somatostatin distributes in hypothalamus with the highest concentration. The somatostatinergic nerve fibers arising from hypothalamus end in dorsal nuclei of vagus to inhibit their activities, thus regulating gastric acid secretion[13]. TCV is adjacent to hypothalamus and has complicated uptake mechanisms on its wall[18]. These morphological characteristics provide the evidence that TCV-injected Oct exerts central inhibitory effect on gastric acid secretion.

Oct injected into TCV of the rat could suppress gastric acid secretion induced by G-5 at the dose of 0.05 μg other than 0.025 μg (Table 2). As the dose increased to 0.1 μg, such inhibition was increased, suggesting that the inhibitory effect of TCV-administrated Oct on the gastric acid secretion is dose-dependent.

Moreover, Pless *et al*[19] used ^{125}I-Tyr^{3}-Oct to investigate the distribution pattern of the central somatostatin receptors and found that the results are similar to those observed using ^{125}I-Tyr^{11}-somatostatin, suggesting that non-specific combination of Oct in the brain does not exist. Based on the above evidence, it is concluded that the central inhibition of gastric acid secretion by TCV-administered Oct is likely to be specific.

In conclusion, Oct injected into the TCV inhibits gastrin-induced gastric acid secretion of rats in a dose-dependent manner. The exact mechanism underlying this effect needs to be further investigated.

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RAPID COMMUNICATION

Clinico-pathological aspects of colorectal serrated adenomas

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Abstract

AIM: To study the association of colorectal serrated adenomas (SAs) with invasive carcinoma, local recurrence, synchronicity and metachronicity of lesions.

METHODS: A total of 4536 polyps from 1096 patients over an eight-year period (1987-1995) were retrospectively examined. Adenomas showing at least 50% of serrated architecture were called SAs by three reviewing pathologists.

RESULTS: Ninety-one (2%) of all polyps were called SAs, which were found in 46 patients. Invasive carcinomas were seen in 3 out of 46 (6.4%) patients, of whom one was a case of familial adenomatous polyposis (FAP). A male preponderance was noted and features of a mild degree of dysplasia were seen in majority (n=75, 83%) of serrated adenomas. Follow-up ranged 1-12 years with a mean time of 5.75 years. Recurrences of SAs were seen in 3 (6.4%) cases, synchronous SAs in 16 (34.8%) cases and metachronous SAs in 9 (19.6%) cases.

CONCLUSION: Invasive carcinoma arising in serrated adenoma is rare, accounting for 2 (4.3%) cases studied in this series.

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Key words: Serrated adenoma; Carcinoma; Polyps; Colorectum

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INTRODUCTION

There has been a steadily increasing interest in serrated adenomas (SAs) of the colorectal mucosa since one of their earliest descriptions by Urbanski *et al*[1] in 1984 and later by Longacre and Fenoglio-Preiser in 1990[2]. As currently understood, SAs have characteristics combining those of hyperplastic polyps and adenomatous polyps. Architecturally, they resemble hyperplastic polyps with a characteristic "saw-toothed" or serrated morphology but their lining epithelium is dysplastic with varying grades of dysplasia (mild, moderate and severe) as seen in adenomatous polyps[3]. Earlier studies have focused on assessing not only their morphology[4-9] and progression[8-12] to carcinomas but also cytogenetic abnormalities which may occur within them[13-16]. This has given rise to the concept of a 'serrated neoplasia pathway' which refers to a pattern of progression of serrated adenomas to carcinomas[4]. The aim of this paper was to study the clinical significance of colorectal SAs with respect to their association with invasive carcinoma, local recurrence, synchronicity, and metachronicity.

MATERIALS AND METHODS

Materials

An archival series of 4536 polyps spanning an 8-year period (1987-1995) were examined retrospectively. To date, this is the largest series of polyps evaluated for serrated adenomas. The adenomas were selected from the St Marks' s database using a computer search for cases which were diagnostically coded as 'colorectal polyps'. The lesions represented polyps from a series of 1096 patients including endoscopically resected polyps and those from resected colons of patients undergoing surgery for carcinoma, FAP, inflammatory bowel disease or diverticular disease.

Methods

The slides (H & E staining) were reviewed by three pathologists (AC, AC & ICT) and all polyps were recorded as hyperplastic polyps, and tubular, tubulovillous, villous adenomas as well as invasive carcinoma. Degree of dysplasia of all polyps was also recorded as being mild, moderate or severe. The histologic diagnosis of serrated adenoma was based on the criteria described by Longacre and Fenoglio-Preiser[2]. In brief, histologic confirmation

Table 1 Details of the 4536 polyps studied

Types of adenoma	*n* (%)
Tubular adenoma	
Mild	3582 (78.8)
Moderate	114 (2.5)
Severe	18 (0.4)
Tubulovillous adenomas	
Mild	87 (1.9)
Moderate	32 (0.7)
Severe	5 (0.1)
Villous adenomas	
Mild	5 (0.1)
Moderate	0 (0)
Severe	0 (0)
Hyperplastic adenomas	600 (13.5)
Serrated adenomas	91 (2)
Invasive carcinoma	14 (1.3)

of a serrated glandular pattern simulating hyperplasia, the presence of goblet cell immaturity, upper crypt zone mitosis, and the prominence of nuclei were the criteria for inclusion. Adenomas showing at least 50% of these features were recorded as SAs. Case notes for patients with SAs were reviewed to record their age, sex, clinical presentation as well as the site, size, number of SAs and their clinical follow-up. All preceding and subsequent polyps were reviewed for the presence of synchronous and metachronous SAs, local recurrence and association with invasive carcinoma. Coexisting diseases were noted, including familial adenomatous polyposis (FAP), hereditary non-polyposis colon cancer (HNPCC), inflammatory bowel disease (IBD), diverticular disease, family history of sporadic colorectal cancer and presence of other polyps. Unlike an earlier study[3], patients with known malignant potential (FAP, HNPCC) and inflammatory bowel disease were not excluded from this study as we wanted to draw attention to SAs arising in these conditions.

RESULTS

SAs were seen to comprise 2% of all polyps ($n=91$) in this series involving 46 (4.2%) patients. Details of the different types of polyps encountered in this study are shown in Table 1.

It was interesting to note that a significant proportion ($n=14$, 15.4%) of SAs were originally reported as tubulovillous adenomas with the majority ($n=75$, 83%) showing a mild degree of dysplasia. There was a male preponderance (70%), with the average age at diagnosis being 65.5 years for males and 67 years for females. The most common presenting complaint was bleeding per rectum. Clinical associations with other diseases in patients with SAs are shown in Figure 1. As seen in the figure presence of previous polyps, FAP and colorectal carcinoma were the conditions which are most frequently associated with SAs.

The most common site of occurrence was the rectum (33%) followed by sigmoid colon (20%). The size of the SAs was seen to vary between 0.1cm and 5.5 cm. The

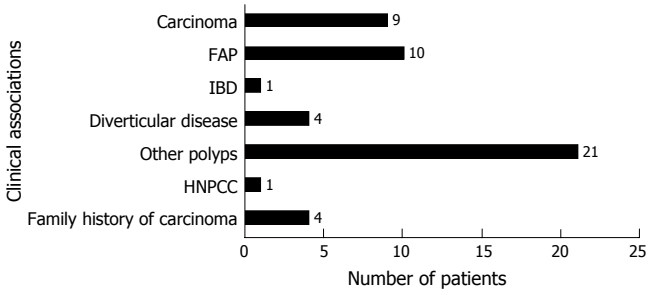


Figure 1 Clinical association of other conditions with serrated adenomas.

total number of synchronous SAs seen per patient varied between 1 (52%, $n=24$) and 8 (2%, $n=1$). Two SAs were seen in 8 (17%), 3 in 9 (20%) and 4 in 4 (9%) patients, respectively.

The mean time of follow-up was 5.75 years (range 1-12 years) with two patients being discharged from follow-up. Five patients died in the course of the follow-up of which 2 were from a FAP-related carcinoma and the remaining 3 were from unrelated causes.

Nine out of 46 (19.6%) patients with SAs had coexisting invasive carcinoma. In 3 (6.4%) cases, invasive carcinoma was seen to arise in SAs. Of these cases, 2 (4.3%) were sporadic and 1 (2.1%) was in a case of FAP. The remaining 6 (13.2%) carcinomas were presented discrete from SAs. The sporadic carcinoma in one of the above two patients was arising in a focus of severe dysplasia within SAs. The lesion was completely excised locally. There was also a synchronous SA in this patient. There was no recurrence at follow-up for 7 years in 2000. In the other patient, the invasive carcinoma arising in SAs was diagnosed in 1994. Local recurrences of invasive carcinoma were seen in 1995 and 1996 after which the patient was lost to follow-up.

All preceding and subsequent polyps retrieved from patients with SAs were reviewed with regard to metachronous SA and local recurrence. Twelve out of 46 (26%) cases showed either a recurrence or metachronicity. In 3 out of the 46 cases (6.5%) there was local recurrence, 2 of these within 1 year. In the remaining 9 cases of the 46 patients (19.6%) with SA, there were metachronous lesions seen. The time interval ranged 1-15 years with a mean time of 6.4 years and a median time of 6 years. Synchronous lesions were seen in 16 out of 46 patients (34.8%) with up to 5 synchronous lesions being present in one case.

DISCUSSION

Our interest in SAs was to evaluate clinically relevant information with respect to their association with invasive carcinoma, local recurrences and metachronicity. The prevalence of SA in our large random sample was 2% which is in the same range with other similar studies where the prevalence was noted to be 1.3%-7%[3,5-7,10] which is lower than 0.5% as originally quoted by Fenoglio-Preiser[2].

Recurrences of SA were seen in 3 cases (6.5%) and probably related to incomplete excision, as dysplastic epithelium was present at the margins of excision histologically. This is however lower than that for other

colorectal polyps which are estimated to be 21%-41% with an average follow-up time of 5-10 years[17]. It must be borne in mind that the apparent difference in the recurrence rates may reflect the difference in the incidence of SA and other polyps. Interestingly, all 3 recurrences were associated with large rectal SAs (3.5 cm and 5 cm in diameter) and took place within 1 year. Synchronicity was noted in 34.8% ($n = 16$) of the cases which are comparable to 20%-61% seen with other adenomas[17] and metachronous SAs were seen in 9 cases (19.6%) which are lower than that quoted for other polyps (32%-55%)[17]. Nevertheless, it implies the real possibility of further SA present in a patient with a histologically confirmed SA. We found serrated adenomas in 10 cases of FAP, 4 cases of IBD and 1 of HNPCC. Though SA may be a feature of FAP, it is not characteristic of the attenuated phenotype with only a few cases being reported in the literature[18].

Progression of SA to frank carcinoma has been suggested in individual cases, but the prevalence of carcinoma originating from serrated adenomas and their cytogenetic characteristics are not fully understood[11,12]. There are a few immunohistochemical studies linking different genetic mutations and oncogene expression in SA[6, 13-15], which may help to reinforce the concept of a "serrated neoplasia pathway"[4].

Inspite of the large number of polyps reviewed in our study, the number of cases in which sporadic cancers were seen to arise remains very small (2 out of 46, 4.3%). The prevalence of invasive carcinoma arising in SA in our study was lower than that in tubular adenomas (4.8%), tubulovillous adenomas (22.5%) and villous adenomas (40.7%) as reported in an earlier series from St Mark's[17]. This prevalence (4.3%) shares some similarity to a large Finnish series which looked at specimens of 466 invasive colorectal carcinomas and found that 27 cases (5.8%) are associated with an adjacent SA[11].

In conclusion, the behaviour of carcinomas arising in SA cannot be reliably differentiated from those arising in other adenomas, given their small number in this series and further work both at identifying morphological features and genetic similarities to carcinomas is needed. SAs do not differ significantly from other adenomas with respect to patient demographics and synchronicity, however they have a lower but a definite incidence of metachronous lesions. Though the prevalence of invasive carcinoma arising in SA was lower than that in tubular, tubulovillous and villous adenomas in our study, further evaluation of this is needed, both of histological and of cytogenetic characteristics. It would be interesting to study SAs in other settings (IBD and polyposis) to shed further light on their behaviour particularly in view of growing evidence of a 'serrated pathway'. The existence of such a distinct pathway of colorectal carcinogenesis has implication most notably for pathologists but at the same time for clinicians who need to recognise the entity of serrated adenomas and its associated potential for neoplastic progression.

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RAPID COMMUNICATION

Platelet-activating factor in cirrhotic liver and hepatocellular carcinoma

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Abstract

AIM: Platelet-activating factor (PAF) is a pro-inflammatory and angiogenic lipid mediator. Here we aimed to investigate levels of PAF, lyso-PAF (the PAF precursor), phospholipase A_2 (PLA_2, the enzymatic activity generating lyso-PAF), acetylhydrolase activity (AHA, the PAF degrading enzyme) and PAF receptor (PAF-R) transcripts in cirrhotic liver and hepatocellular carcinoma (HCC).

METHODS: Twenty-nine patients with HCC were enrolled in this study. Cirrhosis was present in fourteen patients and seven had no liver disease. Tissue PAF levels were investigated by a platelet-aggregation assay. Lyso-PAF was assessed after its chemical acetylation into PAF. AHA was determined by degradation of [^{3}H]-PAF. PLA_2 levels were assessed by EIA. PAF-R transcripts were investigated using RT-PCR.

RESULTS: Elevated amounts of PAF and PAF-R transcripts 1 (leukocyte-type) were found in cirrhotic tissues as compared with non-cirrhotic ones. Higher amounts of PAF and PAF-R transcripts 1 and 2 (tissue-type) were found in HCC tissues as compared with non-tumor tissues. PLA_2, lyso-PAF and AHA levels were not changed in cirrhotic tissues and HCC.

CONCLUSION: While the role of PAF is currently unknown in liver physiology, this study suggests its potential involvement in the inflammatory network found in the cirrhotic liver and in the angiogenic response during HCC.

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Key words: Hepatocellular carcinoma; Cirrhosis; Platelet-activating factor; PAF receptors

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INTRODUCTION

Platelet-activating factor (PAF) is a potent pro-inflammatory lipid mediator that sparks a wide range of immunoregulatory actions[1]. A phospholipase A_2 (PLA_2)-dependent process on membrane alkyl-acyl-glycerophosphocholines generates the lyso-PAF precursor, and its subsequent acetylation results in the PAF molecule[2]. Tissue PAF concentrations are physiologically down-regulated by a specific PAF acetylhydrolase activity (AHA)[2]. PAF acts through specific PAF-receptors (PAF-R) present on the membrane of responsive cells[3]. Several experimental animal models have highlighted that the liver PAF content was elevated by various types of injury, including endotoxin exposure[4,5], ischemia-reperfusion[6], hemorrhagic shock[7], and carbon tetrachloride (CCl_4)-induced cirrhosis[8,9]; results of all these animal studies leading to the conclusions that PAF is a potent vasoconstrictor and systemic vasodilator. While animal models have reported the involvement of PAF in experimental cirrhosis, no clinical study has directly investigated its values in the human cirrhosis liver.

Several *in vitro* studies have highlighted the potential involvement of PAF in carcinogenesis. Thus, PAF acts on the growth of various human tumor cell lines [10,11], increases adhesiveness of tumor cells to vascular endothelia[12], enhances oncogene expression[13], and can contribute to tumor development by enhancing cell motility and by stimulating the angiogenic response[14,15]. Clinical studies have reported elevated PAF amounts in human breast[16], and colon carcinoma[17,18]. However, PAF was not found in higher amounts in lung[19], and thyroid carcinoma[20], suggesting that over-expression of PAF is not necessarily a universal event in carcinogenesis but may be tumor-specific. At present, to our knowledge,

no study has investigated PAF levels in hepatocellular carcinoma (HCC). HCC is a hypervascular tumor. Several angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietins, have been reported to promote angiogenesis in HCC[21]. Of interest, PAF is known to mediate the effect of these angiogenic growth factors in several *in vitro* and *in vivo* models[22-24], suggesting a putative involvement of PAF in HCC.

To improve our knowledge concerning the role of PAF in the human liver, we investigated the levels of PAF and lyso-PAF, the enzymatic activities implicated in PAF production (i.e., PLA_2) and degradation (i.e., AHA) and the presence of PAF-R transcripts in human cirrhotic and HCC tissues.

MATERIALS AND METHODS

Subjects

The procedure of the present study followed the general rules edited by the French National Ethics. Between January 1999 and December 2004, 29 consecutive patients (27 men and 2 females; mean age 62.5 years (range 30-78 years), median age 68 years) with HCC underwent resection in the Department of Surgery of Limoges' CHU. Resectability was preoperatively assessed by ultrasonography, computed tomography and/or magnetic resonance imaging. Intraoperative ultrasonography was performed in all patients. The operative procedures included 4 wedge resections, 10 segmentectomies, 7 left hepatectomies, 6 right hepatectomies and 2 total hepatectomies before liver transplantations. A curative resection, defined as an operation in which all the tumors were macroscopically resected during surgery, was performed in 21 patients (72%). The mean margin from the edge of the tumor was 7.5 ± 1.5 mm. The mean and median tumor size was 7.79 ± 4.6 cm and 8.2 cm, respectively. Twenty tumors (69%) were solitary. Cirrhosis was present in 14 patients (48%), with the cause being alcoholism in 46%, hepatitis C in 35%, hepatitis B in 5%, alcohol and hepatitis in 7%, and unknown in 7%. Two patients had liver fibrosis without cirrhosis, 6 had steatosis, and 7 had no liver disease. Histologically, there were 10 well differentiated HCC (34%), 14 moderately differentiated HCC (48%), 4 poorly differentiated HCC (14%), and 1 fibrolamellar carcinoma, according the histopathologic criteria cited by Kojiro M[25]. Thirteen HCC (44%) had a fibrous capsule that was invaded in 68%. Vascular invasion was present in 19 cases (65%) and 8 HCC presented a high mitosis index. According to pT staging[26], there were 2 pT1, 8 pT2, 13 pT3 and 6 pT4 HCCs. Specimens for the pathologic tissue and the control tissue close to the pathologic one were obtained during the surgical procedure. Specimens were frozen at -80 ℃ until used.

Platelet-activating factor assay

Tissue samples were ethanol-extracted (80% final), purified using thin layer chromatography (TLC), and assayed for PAF activity by aggregation of washed rabbit platelets as previously reported[17-20]. The aggregating activity of samples was measured using a calibration curve obtained with 2.5 to 20 pg of synthetic PAF (Novabiochem, Switzerland). Results were expressed as picograms of PAF per mg of tissue. The lipid compound extracted from blood was further characterised on the basis of its aggregating activity in the presence of 0.1 mmol/L BN 52021 (Tebu, Le Perray-en-Yvelines, France), a specific PAF receptor antagonist and its retention time during TLC.

Assay of lyso-PAF

Lyso-PAF was measured in ethanolic biopsy sample after its chemical acetylation into PAF as previously described[17-20]. Briefly, ethanolic samples were dried, mixed with 200 μL of pyridine and 200 μL of acetic anhydride, and kept overnight in the dark at room temperature. After evaporation of the reagents and removing of the traces of pyridine with chloroform, the dried samples were retrieved with 100 μL of 600 mL/L ethanol, and PAF was bioassayed as described before. The amount of lyso-PAF was established as the difference between the quantity of PAF measured before and after acetylation of the samples. Results were expressed as picograms of PAF per mg of tissue.

Acetylhydrolase assay

Frozen biopsy specimens were pulverised and homogenised in 1 mL of AHA buffer (140 mmol/L NaCl, 3 mmol/L KCl, 4 mmol/L HEPES, 22 mmol/L EDTA). After centrifugation, supernatants were used for AHA and PLA_2 determinations. AHA was assessed as previously described[17-20]. Briefly, 10^5 dpm of [^{3}H]acetyl-PAF (10 Ci/mmol, NEN), 0.1 mmol/L PAF, AHA buffer (pH 8) in a final volume of 450 μL, and 50 μL of tissue extract supernatants were incubated for 30 min at 37 ℃. The reaction was stopped with addition of 100 μL of bovine serum albumin (100 g/L) and 400 μL of trichloracetic acid (200 g/L). Samples were centrifuged at 1500 g for 15 min, and supernatants were counted in a liquid scintillation counter. Results were expressed as fentomoles of PAF degraded per min per mg of tissue as means of duplicate assays. Variation between duplicates was less than 7%.

PLA_2 activity assay

PLA_2 levels were assessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems Europe, Oxon, UK) and as previously described[17,18,20]. Results were expressed as international unit (IU) per mg of tissue as means of duplicate assays. Variation between duplicates was less than 6%

Reverse-transcriptase polymerase chain reaction (RT-PCR) of PAF-R transcripts

Total RNA from tissue samples extracted with RNAwiz (Ambion, Austin, TX) was reverse-transcribed and the complementary DNA (cDNA) was amplified by PCR as previously described[17,18,20]. The human PAF-R transcript 1 sense primer was 5'-GACAGCATAGAG

GCTGAGGC-3', the transcript 2 sense primer was 5' -CCTGAGCTCCCCGAGAAGTCA-3' and the antisense primer was 5'-TAGCCATTAGCAATGACCCC-3. The sense and anti-sense primers of glyceraldehyde-3-phosphate dehydrogenase (GADPH, a positive control of PCR amplification) were 5' -GGCTGAGAACGGGAAGCTTG-3' and 5'-GGATG ATGTTCTGGAGAGCC-3', respectively. PCR products were electrophoresed on a 20 g/L agarose gel (Gibco, France) and visualised by ethidium bromide staining. Expected sizes of amplified products were: PAF-R transcript 1225 bp; PAF-R transcript 2269 bp; spliced variant of PAF-R transcript 2351 bp; and GAPDG, 439 bp.

Statistical analysis

Differences between groups were assessed using the Mann-Whitney *U*-test. A paired student's *t*-test was used to analyse intragroup differences. $P < 0.05$ was considered statistically significant.

RESULTS

PAF and human cirrhosis

A 4-fold increase of PAF levels was found in the cirrhotic tissue (1.61 ± 0.43 pg/mg, $n = 14$) as compared to the non-cirrhotic one (0.39 ± 0.39 pg/mg, $n = 7$) (Figure 1A). In contrast, the AHA levels (the PAF degrading enzyme) were unchanged ($P = 0.33$, Mann-Whitney *U*-test) in the cirrhotic tissue (3.93 ± 0.38 fmol/(min.mg), $n = 14$) as compared to the non-cirrhotic tissue (5.00 ± 0.53 fmol/(min.mg), $n = 7$) (Figure 1B). Similarly, PLA_2 levels (the enzymatic activity that generated the lyso-PAF precursor) were unchanged ($P = 0.23$, Mann-Whitney *U*-test) in the cirrhotic tissue (1.35 ± 0.17 U/mg, $n = 14$) as compared with non-cirrhotic one (1.76 ± 0.24 U/mg, $n = 7$) (Figure 1C). In agreement with these latter results, amounts of the lyso-PAF were similar ($P = 1$, Mann-Whitney *U*-test) in the cirrhotic tissue (1 934.0 ± 556.9 pg/mg, $n = 14$) as compared to the non-cirrhotic one (1 488.9 ± 331.0 pg/mg, $n = 7$) (Figure 1D).

PAF-R and human cirrhosis

The PAF-R gene produces three different species of mRNA [i.e., transcript 1 (leucocyte-type), transcript 2 (tissue-type) and an elongated form of the transcript 2]; both transcripts ultimately yield the functional PAF-R. As shown in Figure 1 (lower panel), RT-PCR experiments showed the presence of PAF-R transcript 1 but not transcript 2 in the cirrhotic tissue.

PAF and human HCC

A significant ($P = 0.0002$, *t*-test for paired data) increase of PAF levels was found in tumor tissues (1.69 ± 0.34 pg/mg, $n = 21$) as compared to the non-tumor ones (1.20 ± 0.33 pg/mg, $n = 21$) (Figure 2A). This increase was significant ($P = 0.0044$, student *t*-test for paired data) for cirrhotic patients (1.88 ± 0.41 pg/mg *vs* 1.61 ± 0.43 pg/mg for tumor and non-tumor tissues, respectively) and also ($P < 0.05$, student *t*-test for paired data) for non-cirrhotic patients (1.29 ± 0.62 pg/mg *vs* 0.39 ± 0.30 pg/mg for tumor and non-tumor tissues, respectively). For HCC patients, PAF values were not significantly different ($P = 0.52$, Mann-Whitney *U* test) according to the tumor status (1.85 ± 0.49 pg/mg *vs* 1.62 ± 0.44 pg/mg for six T1/T2 patients and fifteen T3/T4 patients, respectively). Only tumor PAF levels were affected in HCC tissue. Indeed, the enzymatic activities implicated in PAF production and degradation were not changed. Thus, the AHA levels (the PAF degrading enzyme) were unchanged ($P = 0.24$, *t*-test for paired data) in the tumor tissue (4.72 ± 0.52 fmol/(min.mg), $n = 21$) as compared to the adjacent tissue (4.29 ± 0.32 fmol/(min.mg), $n = 21$) (Figure 1B). Amounts of the lyso-PAF were unchanged ($P = 0.48$, *t*-test for paired data) in the tumor tissue (1 588.9 ± 377.9 pg/mg, $n = 21$) as compared to the non-tumor one (1 616.8 ± 373.7 pg/mg, $n = 21$) (Figure 1D). Moreover, PLA_2 levels (the enzymatic activity that generated the lyso-PAF) were unchanged ($P = 0.26$, *t*-test for paired data) in HCC tissue (1.28 ± 0.27 U/mg, $n = 21$) as compared with non-tumor one (1.49 ± 0.14 U/mg, $n = 21$) (Figure 1C).

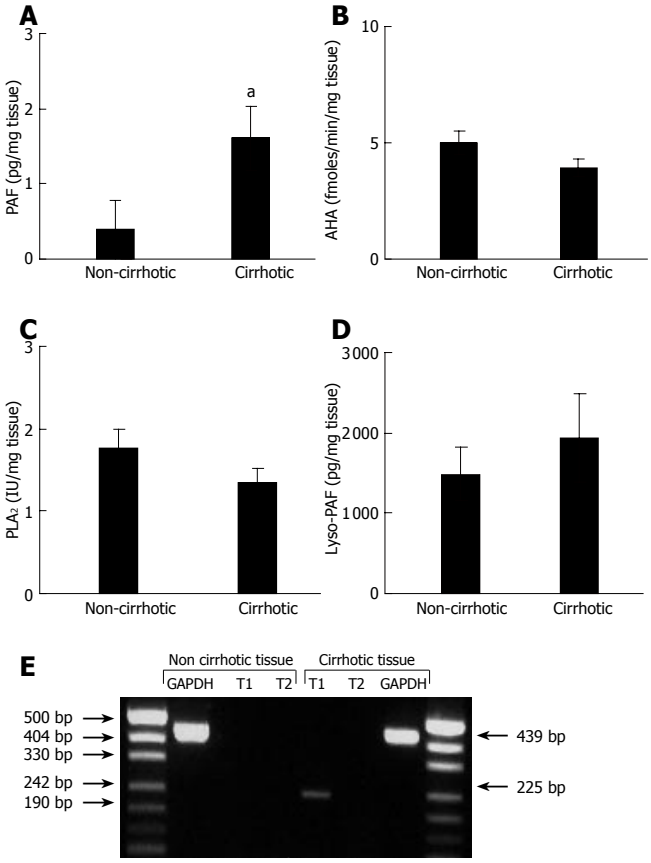


Figure 1 PAF, lyso-PAF, AHA, PLA_2 levels and PAF-R transcripts in cirrhotic and non-cirrhotic liver tissues. *Upper panel* shows the specimens of cirrhotic ($n = 14$) and non-cirrhotic tissue ($n = 7$) obtained during the surgical procedure. **A**: PAF determined by a platelet-aggregation assay, $^aP < 0.05$ *vs* non-cirrhotic; **B**: AHA determined by investigating the degradation of [^{3}H]-PAF, ($P = 0.33$); **C**: PLA_2 assessed by enzyme-linked immunosorbent assay, ($P = 0.23$); **D**: Lyso-PAF measured after its chemical acetylation into PAF, ($P = 1.00$). Results are expressed as mean±SE. Statistical analysis was performed using the Mann Whitney *U*-test. *Lower panel* shows PAF-R transcripts determined by RT-PCR. Expected sizes of amplified products were 225 bp (PAF-R transcript 1), 269 bp (PAF-R transcript 2), 351 bp (spliced variant of PAF-R transcript 2), and 439 bp (GAPDG). Sizes of PCR products and DNA size ladder are indicated by arrows. All experiments were performed in triplicate.

PAF-R and human HCC

As shown in Figure 2 (lower panel), RT-PCR experiments indicated the presence of PAF-R transcripts 1 and 2 in HCC tissues. The spliced variant of PAF-R transcript 2 was not detected.

DISCUSSION

Elevated PAF levels were found in the cirrhotic tissue as compared to the non-cirrhotic one, confirming, for the first time in human, the results obtained in experimental models of liver cirrhosis[8,9]. Elevated levels of PAF can result from a higher PAF production or a lower PAF degradation. Arguing against the latter hypothesis, the AHA levels (the PAF degrading enzyme) were unchanged in the cirrhotic tissue as compared to the non-cirrhotic tissue. Thus, the elevated PAF levels in cirrhotic tissue might be due to an elevated PAF synthesis. Kupffer cells which are a well known source of PAF[27] might be an appropriate candidate for these elevated tissue PAF levels. Strengthening this hypothesis, Kupffer cells from cirrhotic rat was found to produce more PAF than kupffer cells of control rats[8]. Confirming a previous study[28], PLA_2 levels (the enzymatic activity that generated the lyso-PAF) were unchanged in the cirrhotic tissue. In agreement with these latter results, amounts of the lyso-PAF were similar in the cirrhotic tissue as compared to the non-cirrhotic one. PAF acts as a local cell-to-cell mediator. Thus, an elevated PAF synthesis is only of relevance if PAF-R can be detected at the site of PAF production. The PAF-R gene produces three different species of mRNA (i.e., transcript 1, transcript 2 and an elongated form of the transcript 2); both transcripts ultimately yield the functional PAF-R[3]. Leucocytes produce PAF-R transcripts 1, while PAF-R transcripts 2 are found in organ extracts. Thus, PAF-R transcripts 1 and 2 are also named "leukocyte-type" and "tissue-type", respectively. RT-PCR experiments indicated the presence of PAF-R transcript 1 in the cirrhotic tissue. These PAF-R transcripts from the "leukocyte-type" fit well with the previous study that showed higher hepatic levels of PAF-R mRNA in CCl_4-induced liver cirrhosis[8], with cirrhotic kupffer cells as cellular source of these PAF-R mRNA. Thus our present study concludes that PAF and PAF-R transcripts are present in the human cirrhotic liver. PAF may have pathophysiologic functions and might account, at least in part, in the inflammatory response occurring in the cirrhotic liver. On the one hand, PAF might act directly by stimulating the production of humoral mediators including peptide leukotrienes (LTC_4, LTD_4, LTE_4)[29], prostaglandin E_2 (PGE_2)[30], and nitric oxide[31], which can affect cell proliferation and gene expression. On the other hand, and with respect to its potent immunoregulatory actions[1,32], PAF might act indirectly on stellate cell-immune cell interactions by promoting leukocyte chemotaxis and adherence, by influencing leukocyte activation and by affecting their cytokine secretions.

Previous clinical studies have reported the elevated PAF levels in breast and colon carcinomas[16-18], but not in lung and thyroid carcinomas[19,20], suggesting that over-expression of PAF is not necessarily a universal event in carcinogenesis, but may be tumor-specific. Increased PAF levels were found in HCC tissues. In contrast to colorectal cancer patients[17], tumor PAF level did not change according to the tumor status. As suggested above, the elevated PAF levels in HCC tissue might be due to elevated PAF synthesis by Kupffer cells. In contrast to that found in colon carcinoma[17], only tumor PAF levels were affected in HCC tissue. Indeed, the enzymatic activities implicated in PAF degradation (i.e., AHA) and production (i.e., PLA_2) were not changed; these latter results fitting well with the data reporting no differences in phospholipids composition (including phosphatidylcholine one) between human hepatoma homogenates and normal regions outside the tumors[33]. Few clinical studies had focussed on PAF-R levels in cancer tissue. Levels of PAF-R transcripts were not modified in colorectal, lung and thyroid carcinoma[17,19,20], while PAF-R (tissue-type) levels were increased in liver metastasis of colorectal cancer[18]. PAF-R transcripts 1 (leukocyte-type) and 2 (tissue-type) were found in the HCC tissues. Kupffer cells might be the cell source for the elevated levels of PAF-R

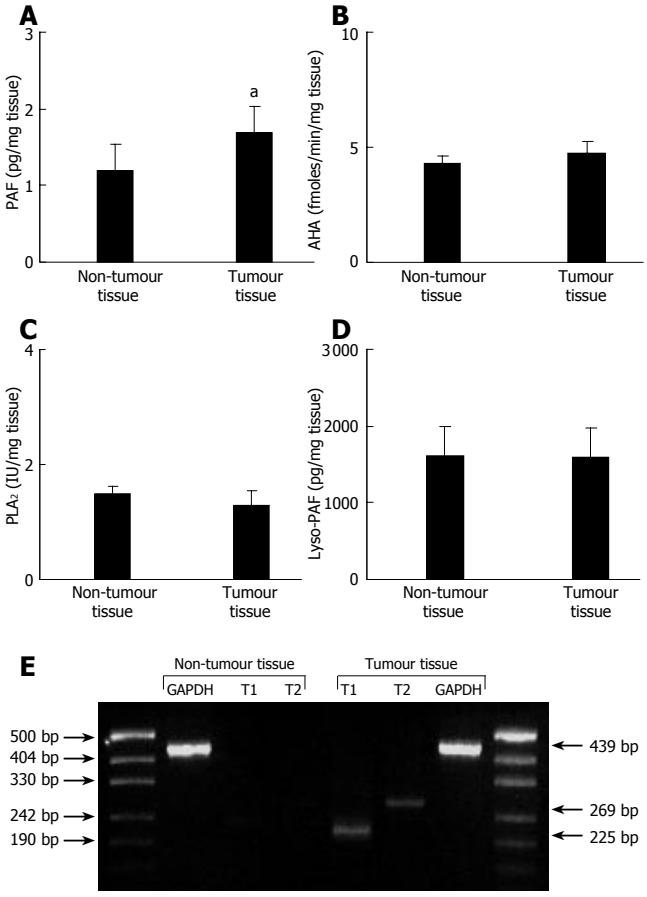


Figure 2 PAF, lyso-PAF, AHA, PLA_2 levels and PAF-R transcripts in HCC and non-tumour tissues. *Upper panel* shows specimens from 21 HCC patients obtained during the surgical procedure. **A**: PAF, $^aP < 0.05$ *vs* non-tumour tissue; **B**: AHA ($P = 0.24$); **C**: PLA2 ($P = 0.26$); **D**: Lyso-PAF ($P = 0.48$). Statistical analysis was performed using the Students' t-test for paired samples. Results are expressed as mean ± SE. *Lower panel* shows PAF-R transcripts determined by RT-PCR. Expected sizes of amplified products were 225 bp (PAF-R transcript 1), 269 bp (PAF-R transcript 2), 351 bp (spliced variant of PAF-R transcript 2), and 439 bp (GAPDG). Sizes of PCR products and DNA size ladder are indicated by arrows. All experiments were performed in triplicate.

(leukocyte-type) transcripts. Thus, transforming growth factor beta (TGF-β) and PAF stimulated PAF-R mRNA synthesis in monocytic/macrophagic cells[34,35]. Elevated levels of TGFβ[36] and PAF (Figure 2A) are found in HCC tissues. PAF-R transcript 2 (tissue-type) has also been reported to be up-regulated in HCC tissue. Such an up-regulation of PAF-R transcripts 2 has been previously reported in endometrial cells in response to estradiol[37]. Interestingly, locally elevated estrogen formation has been reported in HCC tissues[38], and estrogen enhances angiogenesis through a pathway involving PAF in a mouse Matrigel model *in vivo*[39]. Thus, whatever molecular signals implicated in these elevated PAF-R transcript levels, the current data argue for a pathophysiological role of PAF in HCC tissues.

In conclusion, PAF is a potent pro-inflammatory compound that acts as a local cell-to-cell mediator. Since PAF can generate biological responses detectable at levels of 10 fmol/L, regulating PAF levels is important since elevated levels of PAF might result in biological effects. To the best of our knowledge, this clinical study, for the first time, reports an increase in PAF and PAF-R transcripts in cirrhotic liver tissue and HCC tissue, suggesting a pathological role for PAF in liver cirrhosis and HCC. In cirrhotic tissue, PAF might act on stellate cell-immune cell interactions by influencing leukocyte activation and chemotaxis, and by altering the local cytokine network. An angiogenic role of PAF might be suggested in HCC.

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RAPID COMMUNICATION

Determination of anti-endomysium IgA antibodies in the diagnosis of celiac disease: Comparison of a novel ELISA-based assay with conventional immunofluorescence

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Abstract

AIM: To evaluate the novel anti-endomysium (anti-EMA) detection based on ELISA.

METHODS: Anti-EMA IgA was measured by a novel ELISA in 196 patients with gastrointestinal symptoms and suspected mal-absorption. Data were compared with those obtained by the conventional IF test.

RESULTS: A good concordance of 98% was found between these two assays. In sera of 161 patients (82%) both assays tested negative whereas in sera of 31 patients (16%) both assays tested positive for the presence of anti-EMA antibodies. Discrepancies between EMA-ELISA and EMA-immunofluorescence (IF) were found in only 4 patients (2%).

CONCLUSION: This ELISA can replace IF for the detection of anti-EMA antibodies and provide clinicians with an excellent tool to screen for celiac disease in patients with gastrointestinal complaints.

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Key words: Celiac disease; Endomysium; ELISA; Immunofluorescence; Histology

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INTRODUCTION

Celiac disease (CD) is the most common cause of intestinal malabsorption in the Western world with a recently described incidence of 1:100 to 1:300. Gastro intestinal damage is the result of gluten-dependent change in villous morphology and/or signs of immunological activation in the lamina propria, which is reversible on a strict gluten-free diet[1]. A biopsy from the proximal small intestine is essential for diagnosis. The number of biopsies however, can be dramatically reduced by using an objective, reliable, and easy to perform serologic screening. The antibodies most commonly detected in clinical routine are anti-tissue-transglutaminase IgA (anti-tTGA) and anti-endomysium IgA (anti-EMA)[2]. Anti-tTGA in combination with anti-EMA antibodies accounts for almost 100% sensitivity and specificity[3,4]. Anti-EMA antibody detection is at present performed with an immunofluorescence (IF) technique on cryostat sections of monkey oesophagus. Anti-tTGA antibody detection is based on an enzyme linked immunosorbent assay (ELISA). Disadvantages of IF are the qualitative results, the high costs, the need for extensively trained personnel and the ethical disapproval[2,3].

Anti-EMA antibody detection based on ELISA overcomes the disadvantages of IF. In this study we evaluated a new ELISA for the detection of anti-EMA antibodies and compared these data with the anti-EMA results obtained by IF.

MATERIALS AND METHODS

The study population consisted of 196 patients (male 35%, female 65%) with gastrointestinal symptoms and suspected mal-absorption, visiting the Department of Internal Medicine or the Department of Pediatrics of the Diaconessenhuis in Leiden or the Albert Schweitzer Hospital in Dordrecht, respectively, between November 2002 and May 2005. Total IgA was determined by immunoturbidimetric assays on the Hitachi-917 (Roche Diagnostics Corporation, Indianapolis, IN, USA) or the Beckmann-immage autoanalyser (Beckman-Coulter, Mijdrecht, the Netherlands).

Detection of anti-EMA antibodies by IF was performed by using cryostat sections of monkey oesophagus as an antigen substrate (The Binding Site,

Table 1 Comparison of the results of anti-EMA antibody detection by IF and ELISA in 196 patients

EMA-IF	EMA-ELISA	*n* (%)	
-	-	161 (82)	no biopsy results
+	+	31 (16)	biopsy: indicative for CD
+	-	3 (1.5)	tTGA -, no biopsy results
-	+	1 (0.5)	anti-EMA 22 U/mL, tTGA -, biopsy: not indicative for CD

- = negative results; + = positive results; *n* = the number of patients.

Birmingham, UK) according to the manufacturer's instructions. All slides were scored by two independent observers and a definite result was obtained by consensus.

Anti-EMA antibodies were also detected with an ELISA (Medipan Diagnostica, Selchow, Germany) according to the manufacturer's instructions (www.genericassays.com). Microplate strips coated with purified human endomysium autoantigens (90-300 kDa) were incubated with 100 μL 1:50 diluted serum, a positive control and a 4-point calibrator curve were included in each run. The strips were incubated for 1h at 37 ℃ and washed six times with 300 μL washing buffer and 100 μL horseradish peroxidase-conjugated anti-human IgA was added. After 30 min incubation at 37 ℃ the plates were washed 6 times, 100 μL of substrate solution containing 3, 3', 5, 5' tetramethylbenzidine was added and the reaction was stopped after 10 min at ambient temperature in the dark with 100 μL 0.25 mol/L H_2SO_4. The absorbance was measured at 450 nm. A standard curve was established by plotting the optical density of each calibrator with respect to the corresponding concentration value in U/mL. From the OD of each sample the corresponding antibody concentration expressed in U/mL could be determined. The cut-off value for positivity was 20 U/mL. Patients who tested positive for serologic tests, underwent a small intestinal biopsy.

RESULTS

None of the patients included in this study showed an IgA deficiency. Sera of 161 patients tested negative for anti-EMA antibodies with both IF and ELISA (Table 1). In sera of 31 patients anti-EMA antibodies were detected with both IF and ELISA and the diagnosis of CD could be confirmed by histological results. In sera of 4 patients discrepancies were found in anti-EMA results between IF and ELISA. In sera of 3 patients the presence of anti-EMA antibodies was only detected with IF. No histological data were available for these 3 patients. In serum of 1 patient the slightly increased anti-EMA concentration (22 U/mL) was only detected with ELISA, and CD could not be confirmed by histological results.

DISCUSSION

To the best of our knowledge, this is the first anti-EMA assay based on endomysium antigens from human origin. The detection of anti-EMA antibodies by an ELISA has been described before[5,6]. This EMA-ELISA however is based on endomysium antigens purified by affinity chromatography from primate liver and is not commercially available anymore.

We compared the EMA-ELISA and the EMA-IF by assaying 196 patients with gastrointestinal symptoms and suspected mal-absorption. A good concordance of 98% was found between both these assays. In sera of 161 patients (82%) both assays tested negative whereas in sera of 31 patients (16%) both assays tested positive for the presence of anti-EMA antibodies and the diagnosis of CD was confirmed by histological results. Discrepancies between EMA-ELISA and EMA-IF were found in only 4 patients (2%). In sera of 3 of these patients the presence of anti-EMA antibodies was detected by IF but not by EMA-ELISA, however in these sera anti-tTGA antibodies were absent (results not shown), suggesting the absence of CD. Since no histological data were available the diagnosis of CD could not be excluded or confirmed. In serum of 1 patient the presence of anti-EMA antibodies detected by ELISA could not be confirmed by IF and anti-tTGA antibodies were absent. The slightly increased anti-EMA antibody concentration (e.g. 22 U/mL) appears to be false positive since the biopsy results were not indicative for CD.

Quantitative results obtained by EMA ELISA are easy to interpret in contrast to qualitative results obtained after IF, providing the clinicians with an excellent tool to screen for CD in patients with gastrointestinal complaints in the absence of IgA deficiency.

In conclusion, this ELISA can replace IF for the detection of anti-EMA antibodies. In daily practice, testing for anti-EMA antibodies is frequently performed in conjunction with testing for anti-tTGA antibodies since this combination accounts for almost 100% sensitivity and specificity[3, 4].

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RAPID COMMUNICATION

Establishment of model of visceral pain due to colorectal distension and its behavioral assessment in rats

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CONCLUSION: Scoring the AWR during colorectal distensions can assess the intensity of noxious visceral stimulus. Flatting of abdomen (AWR 3) to CRD as the visceral pain threshold is clear, constant and reliable. This pain model and its behavioral assessment are good for research on visceral pain and analgesics.

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Key words: Visceral pain; Colorectal distension; Rat behavior

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Abstract

AIM: To establish a visceral pain model *via* colorectal distension (CRD) and to evaluate the efficiency of behavioral responses of CRD by measuring the score of abdominal withdrawal reflex (AWR) in rats.

METHODS: Thirty-eight male SD rats weighing 180-240g were used to establish the visceral pain model. The rat was inserted intra-anally with a 7 cm long flexible latex balloon under ether anesthesia, and colorectal distensions by inflating the balloon with air were made 30 min after recovering from the anesthesia. Five AWR scores (AWR0 to AWR4) were used to assess the intensity of noxious visceral stimuli. It was regarded as the threshold of the minimal pressure (kPa) for abdominal flatting was induced by colorectal distension.

RESULTS: A vigorous AWR to distension of the descending colon and rectum was found in 100% of the awake rats tested. The higher the pressure of distension, the higher the score of AWR. The distension pressures of 0, 2.00, 3.33, 5.33 and 8.00 kPa produced different AWR scores ($P < 0.05$). The pain threshold of AWR was constant for up to 80 min after the initial windup (first 1-3 distensions), the mean threshold was 3.69 ± 0.35 kPa. Systemic administration of morphine sulfate elevated the threshold of visceral pain in a dose-dependent and naloxone reversible manner.

INTRODUCTION

Visceral pain is produced by diseases[1]. It has not yet been studied more than the somatic pain due primarily to the lack of a reliable, stable model of visceral pain. The mechanisms of acute and chronic visceral pain have been extensively studied since the reports from Ness *et al* in 1998[2-4]. But implantation of the electrode in the abdominal muscle to record the electric muscle graph is complicated, and disturbs the movement of the awake experimental animals and increases the somatic hypersensitivity[5]. The practicability of the model is therefore limited. Al-Chaer *et al*[5] have established the abdominal withdrawal reflex (AWR) scores in a model of chronic visceral hypersensitivity in rats. We used AWR scores of the acute colorectal distension to establish a visceral pain model in rats. The minimal pressure for flatting of abdomen induced by CRD was regarded as the threshold for the assessment of the reliability and stability of the model and its behavioral reaction.

MATERIALS AND METHODS

Experiment animals

Male Sprague-Dawley (depurator grade) rats ($n = 38$) weighing 180-240g were obtained from the Experiment Animal Center of Soochow University.

Establishment of CRD

The colorectal distension balloon was made from a latex glove finger attached to an 8# rubber urethral catheter (7 cm in length and 2.5 cm in diameter).

Rats were randomly grouped for the experiment. Rats were deprived of food but had free access to water 24 h before the experiment. The prepared colorectal distension balloon covered with lubricant was totally inserted into the descending colon and rectum under ether anesthesia. Rats were put into a 20 cm×15 cm×15 cm glass box for the experiments 30 min after they were fully recovered from the anesthesia. The tube of balloon was connected *via* a Y connector to a air pump and a sphygmomanometer. The balloon that was inserted into the rat coloratura was inflated with air at a speed of 0.133 kPa/s(1 kPa = 7.5 mmHg), and the pressure inside the balloon was continuously monitored. The distension duration and intervals between two distensions were selected.

Behavioral study

Five abdominal withdrawal reflex (AWR) scores (AWR0 to AWR4) were used to assess the intensity of noxious visceral stimuli: AWR0: remarkable behavior changes; AWR1: immobility of the rat body or occasionally clinches of the head; AWR2: mild abdominal muscle contraction; AWR3: lifting the abdomen off the box platform or flatting of abdomen; AWR4: body arching or lifting pelvic structures off the platform[5]. The cut-off pressure to avoid the rectum injury was set at 13.33 kPa.

The pain threshold was defined as the minimal pressure (kPa) inside the balloon when the rat showed flatting of abdomen (AWR 3) during the colorectal distension (CRD).

Pharmacology study

To observe the effect of morphine on the abdominal withdrawal reflex, 24 rats were divided into 4 groups ($n = 6$ in each group and giving morphine s.c. 1 mg/kg, 2 mg/kg, 4 mg/kg and 8 mg/kg, respectively). Each received colorectal distension once per 5 min. The mean score of the 4th and 5th distension was regarded as the basic value. The pain thresholds at 10, 15, 20 min after morphine administration were recorded. The maximal possible effect (MPE) was used to compare the effect of morphine[6]. Percent maximal possible effect (%MPE) = (post drug value - baseline value)/(cutoff value - baseline value) × 100%[7].

Statistical analysis

Data were presented as mean ± SD. The statistical significance tested by analysis of variance ($P < 0.05$) and *post hoc*. LSD (with comparable variance) or Tamhane's T2 (with incomparable variance) method was selected. All the analyses were performed using SPSS 10.0 software.

RESULTS

Stepping increased pressure of colorectal distension

The scores of abdominal withdrawal reflex were recorded in 8 rats receiving the colorectal distension every 4 min with stepping increased pressures of 0, 2.00, 3.33, 5.33, 8.00 kPa respectively and lasted for 30 s. The mean value of 3 times of abdominal withdrawal reflex was adopted. All the rats showed the abdominal withdrawal reflex to the colorectal distension (Figure 1). The higher the pressure, the higher scores of the abdominal withdrawal reflex. There were statistically significant differences among the five pressure grades.

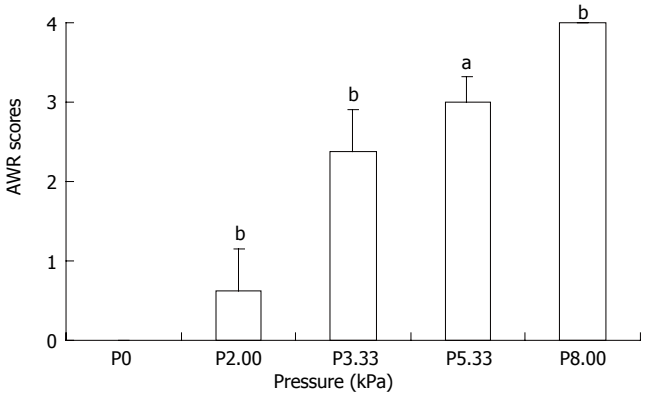


Figure1 AWR scores under stepping increased pressure of colorectal distension $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the basal pressure.

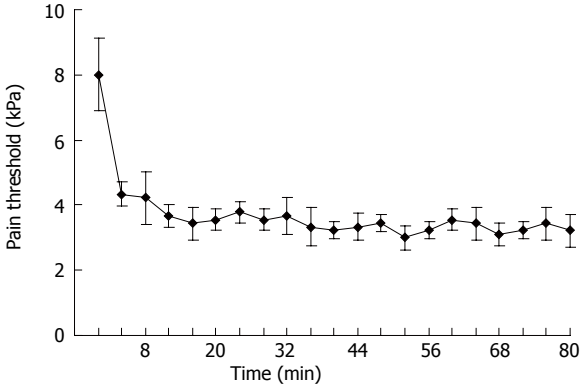


Figure 2 Repeated observations of pain thresholds after CRD at different times. The threshold was higher at the first distention, and then declined. It became stable after 3 trials. The mean pain threshold was 3.69 ± 0.35 kPa.

Repeated observation of pain thresholds after CRD at different times

The pain threshold was measured in 6 rats receiving a colorectal distension per 4 min. The results showed (Figure 2) that the threshold was higher at the first distention and then declined. It became stable after 3 trials. The mean pain threshold was 3.69 ± 0.35 kPa. The pain thresholds were measured on d 5 and 10 after the procedure in the same rats, and the mean thresholds were 3.59 ± 0.22 kPa and 3.63 ± 0.31 kPa with no significant difference among them.

Effect of mophine-naloxone on AWR to CRD

Subcutaneous administration of morphine increased the pain threshold in a dose-dependent manner. The pain thresholds of the rats that received respectively, 2 mg/kg, 4 mg/kg, 8 mg/kg of morphine were significantly higher compared to the basal values ($P < 0.01$). The duration of antinociceptive effect produced by 8 mg/kg morphine

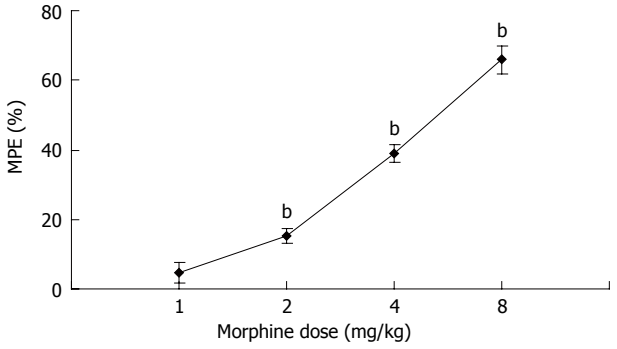


Figure 3 Effect of morphine on AWR to CRD. The pain thresholds of the rats that received 2 mg/kg, 4 mg/kg, 8 mg/kg of morphine respectively were significantly higher than the basal values ($P<0.01$). $^bP<0.01$ *vs* the basal values.

lasted for more than 2.5 h (150.45±22.50 min, Figure 3). Naloxone (0.5 mg/kg, i.p.) 20 min after 4 mg/kg morphine administration increased the movement of rats and the analgesic effect of morphine was totally antagonized. Five min after administration of naloxone, the pain threshold decreased to the basal level.

Anatomic observation

After the distension experiment, rats ($n=6$) with the balloon remained in the coloratura and the pressure inside the balloon kept at 8.00 kPa (AWR 4) were anesthetized with chloral hydrate. Shaped like column, the balloon occupying the whole descending colon and rectum was found in the left hypochondria region after opening the abdomen. The distended balloon was 8.94±1.20 cm in length and 1.20±0.21 cm in diameter. Inflating the balloon at a speed of 0.133 kPa per second, the pressure causing rupture of the colon was 19.80±1.38 kPa. Although the length of the whole colon in rats was 16.90±2.52 cm, the site of the rupture was always in the descending colon.

DISCUSSION

Visceral noxious stimulus similar to the somatic noxious stimulus contributes to the false effective responses in animals, including agony behavior, bray, and response of the circulation and respiration system, muscle contraction, escaping, *etc*. The ideal visceral noxious stimulus with less effect on surrounding tissues should be controllable and repeatable[8,9]. The chemical[10], electrical stimuli[11]on the visceral afferent fibers are the most commonly used methods in research of visceral pain, but the responses to chemical or electrical stimuli are instable and nonspecific[2].

The quantified EMG of abdominal muscles[2], cardiovascular response[2], latency of step-down[12] and abdominal musculature contractions[6] have been adopted for assessment to the stimulus and response intensity, yet these indexes are easily interfered with multi-factor. Behaviors such as immobility of rat body, anus distension, abdominal muscle contraction, back arching, testis lifting, hind leg spreading with gradually increasing the pressure in colorectalregion have been found in rats. If the pressure is persistent such behaviors can last for a longer time. Therefore, the responses to colorectal distension meet the nociceptive pain standards in animal experiment. CRD seems to mimic the pathological mechanisms of pain in humans[9].

The abdominal withdrawal reflex (AWR) is a semi-quantitative, autonomic motor reflex that is similar to visceromotor response (VMR). The reflex arc of abdominal withdrawal reflex involves the supraspinal mechanisms. The scoring of AWR is based on the enhancement of the abdominal muscle contraction, which is free of the direct abdominal muscle stimulation by the electrodes, so the somatic hypersensitivity during the study of visceral pain is eliminated[5]. In the present study, all the rats showed the abdominal withdrawal reflex to the colorectal distention, suggesting that the AWR scores reflect the visceral pain to some extent.

Since the observation of rat body or head movement (AWR 1) and the initiation of abdominal muscle constriction (AWR 2) are difficult, we consider that AWR 3 (lifting the abdomen off the box platform or flatting of abdomen) and AWR4 are more reliable responses of the animals to the visceral pain. The animal's performance of flatting of abdomen (AWR 3) is most clear and exact in response to CRD. If the distention pressure in the balloon does not change, the performance of flatting of abdomen is stable within a few minutes and no significant variations in pain threshold among individuals are noticed. Therefore, the minimal pressure (mmHg) inside the balloon is the most eligible index of the pain threshold when the rat shows flatting of abdomen（AWR 3）during CRD.

In this study, morphine (sc) inhibited the responses to CRD in a dose-dependent manner and this inhibitory effect of morphine could be antagonized by naloxone, demonstrating the stability and reliability of the visceral pain model produced by CRD in rats.

Ness and Gebhart[2] reported that the VMR threshold is 22.4±0.9 mmHg(2.99±0.12 kPa), which is similar to the values of the AWR 2 in the present study. The pain threshold in our study was 3.69±0.35 mmHg (AWR3), which was much lower than that reported by A1-Chaer *et al*[5] [53.5±2.8 mmHg(7.13±0.37 kPa)]. This difference may be due to the different species of the animals and the length of the balloon used. The relationship between the length of balloon and the threshold of distention pressure suggests a possible mechanism of "spatial summation" in visceral pain[9].

In the present study, the visceral pain threshold at the first distention was higher, which might be attributed to the "windup" effect induced by the initial noxious stimuli on visceral fibers[2]. We found that the threshold after the initial 2-3 trials became more stable in the actual visceral pain experiment. It is better to wait 1 or 2 min before the next CRD is given. The feces in the coloratura interfered with the insertion of the balloon and affected the pressure in the colorectum, thus the animals should be deprived of food intake before experiment. The pressure causing the rupture of colon was 16.67 to 21.33 kPa, averaged 19.80± 1.38 kPa. To avoid the injury of the colon during the CRD, the cut-off pressure should be 13.33 mmHg when a 7 cm long balloon is used.

In conclusion, using colorectal distension to establish a model of visceral pain in rats is simple and reliable. The scores of abdominal withdrawal reflex (AWR) to CRD are

good for quantitative behavior assessment. This model can be used in experiments with free movement of animals. It provides a good animal model for study of the mechanism of visceral pain and the effects of different drugs on visceral pain.

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RAPID COMMUNICATION

Generation and characterization of a transgenic mouse model for pancreatic cancer

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Abstract

AIM: To generate a SV40Tag transgenic tumor animal model and to study the mechanism underlying tumorigenesis.

METHODS: A mammary gland expression vector containing SV40Tag DNA was generated. Transgene fragments were microinjeted into fertilized eggs of FVB mice. The genetically manipulated embryos were transferred into the oviducts of pseudo-pregnant female mice. PCR and Northern blot analysis were used for genotype analysis of F1 and F2 mice. Transgene expression was detected by RT-PCR and immunohistochemistry.

RESULTS: SV40Tag gene was detected in two lines of transgenic mice. One of them delivered the transgene to F1 and a tumor was found in the pancreas of these mice. RT-PCR and immunohistochemistry showed that SV40Tag gene was expressed in the tumor. Pathological characterization of the transgenic mice demonstrated that the tumor belonged to pancreatic cystic neoplasm.

CONCLUSION: SV40Tag transgenic mouse model can be successfully established. The transgenic mice develop a pancreatic tumor, which can be used for investigation of the molecular mechanism of tumorigenesis *in vivo*.

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Key words: SV40Tag; Transgenic mice; Animal model; Pancreatic neoplasms

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INTRODUCTION

Pancreatic cancer is one of the most common malignant diseases threatening human life. The survival rate of pancreatic cancer patients is among the lowest in all kinds of cancer patients. The 1- 3- and 5-year survival rates have been reported to be 16%, 5% and 4%, respectively[1]. Early diagnosis and effective therapeutic intervention are essential to improve the situation. Animal models of pancreatic cancer should provide important tool for the identification of biomarkers for early detection and molecular mechanism studies of the pathological procession of pancreatic cancer.

Simian virus 40 T antigen (SV40Tag) is a virus oncogene encoded by simian virus 40 (SV40). SV40Tag protein is a multifunctional regulatory protein that binds to and inactivates tumor suppressor genes, including P53 and Rb. It has been shown that interaction between SV40Tag and tumor suppressor proteins stimulates DNA duplication, thus facilitating cell proliferation and inducing cell transformation[2,3]. A number of tumor transgenic animal models have been established, including pancreatic cancer[2,3], bone sarcoma[4], urinary bladder carcinoma[5] and liver cancer[6]. In this report, we described the generation and characterization of a SV40Tag transgenic mouse model for pancreatic cancer. The animal model may be useful for molecular mechanism studies on this disease.

MATERIALS AND METHODS

Experimental animals

FVB and CD-1 mice were provided by Center of Comparative Medicine of Yangzhou University. Mice were maintained in standard laboratory conditions in a 12h light/12h dark cycle.

Expression vectors

A 2482-bp SV40Tag DNA fragment was generated by PCR using COS-1 genomic DNA as template. The PCR

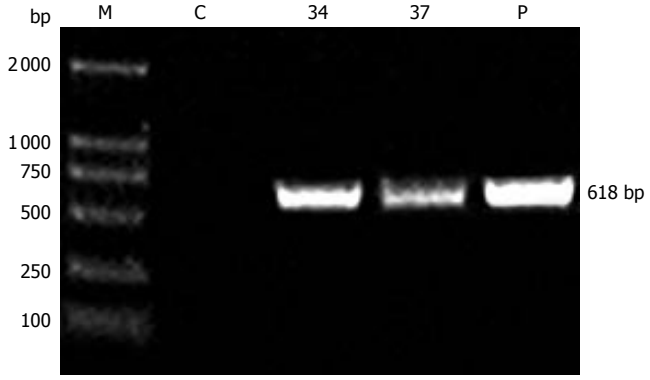


Figure 1 PCR results of transgenic mice. M: DL 2000 marker; C: Non-transgenic mouse concrol; P: pBC-SV40Tag plasmid; 34 and 37: the number of gene positive mice.

product containing the entire SV40Tag coding region and a 346 bp intron with two Xho I sites at both ends was purified, digested with Xho I and then cloned into pBC-1 vector to generate the transgenic construct pBC-SV40Tag.

Preparation of transgenic mice

An 18.2 kb pBC-SV40Tag fragment was purified from agarose gel (Qiagen, CA, USA) and adjusted to a final concentration of 2 μg/mL in PBS. FVB female mice were hormonally super-ovulated and mated with FVB male mice. The fertilized eggs were collected from the oviduct 24 h later. Transgene pBC-SV40Tag in PBS was microinjected into the pronuclei of fertilized eggs. Finally, the injected fertilized eggs were transplanted into the oviduct of pseudo-pregnant CD-1 mice.

Genotype of transgenic mice

Founder (G0) mice were identified by PCR and Southern blotting analysis. Genomic DNA was isolated from the tails of transgenic mice using a standard method. For PCR, a pair of primers spanning SV40Tag introns was designed (forward: 5'-ACTTTGGAGGCTTCTGGGAT-3' and reverse: 5'-GGTGTAAATAGCAAAGCAAGCA-3'), which would produce a 618 bp fragment for genomic DNA template and a 272 bp fragment for cDNA template. PCR using genomic DNA as template was performed under the following condition: 35 cycles at 94℃ for 1 min, at 60℃ 45 s, at 72℃ for 50 s. PCR products were detected by electrophoresis on 1.0 % agarose gels. After PCR screening, transgenic mice were further confirmed by Southern blot analysis. Genomic DNA was digested overnight with Nde I and subjected to electrophoresis on a 0.8 % agarose gels. DNA was transferred onto nylon membrane (Millipore Co., Ltd). The 1 kb fragment of plasmid pBC-SV40Tag digested with Nde I was used as a probe. Southern blot analysis was performed according to ECLTM direct nucleic acid labeling and detection system (Amersham).

Morphology and anatomy examination

Transgenic mice were examined every day. Mice with bulgy belly and showing listlessness were anatomized and photographed. Various organs and tumors were dissected from the transgenic mice and fixed in 10 % formalin. Sections were obtained from paraffin-embedded tissue samples, stained with hematoxylin-eosin, and examined under a microscope and photographed.

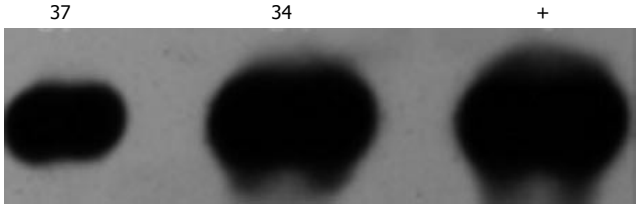


Figure 2 Southern blotting of transgenic mice. +: pBC-SV40Tag plasmid positive control; 34 and 37: the number of gene positive mice.

Expression of transgene

Total RNA was isolated from tumor and other tissues with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized using reverse transcriptase (Promega). RT-PCR was performed using the SV40Tag primers described above. PCR was performed for 35 cycles at 94℃ for 1 min, at 60℃ for 45 s, and at 72℃ for 50 s. The PCR products were analyzed by electrophoresis on 1.0 % agarose gels.

Immunohistochemistry analysis

Tissues expressing SV40Tag were collected and fixed in 10 % formalin. Paraffin-embedded tissue samples were cut into 5μm-thick sections. For immunohistochemistry, tissues were de-waxed and treated with newly diluted 3 % H_2O_2 for 15 min to destroy internal peroxidases. Tissues were incubated with normal goat serum at room temperature for 20 min, then incubated with polyclonal antibody of SV40Tag (Santa cruz biotechnology, sc-20800, 1∶400) at 37℃ for 120 min, and finally with goat anti-rabbit secondary antibodies at 37℃ for 30 min. The samples were stained with DAB using SABC kit (Boster). The nuclei were counterstained with hematoxylin. Slices were observed under microscope and photographed (Olympus).

RESULTS

Establishment of pBC-SV40Tag transgenic mice

pBC-SV40Tag was generated and confirmed by restriction enzyme map and DNA sequence analysis. A total of 39 mice were analyzed for production of transgenic mice. Two mice, one male and one female, were identified as transgenic mice by PCR (Figure 1). The serial numbers of the positive mice were line 34 (female) and line 37 (male). These transgenic mice were confirmed by Southern blotting analysis using Nde I digested SV40Tag DNA fragment as a probe (Figure 2). The line 34 developed a tumor at early stage (about 4 wk). Line 37 produced F1 off-springs by mating with normal FVB female mice.

Tumorigenesis of pBC-SV40Tag transgenic mice

Line 37 mice and positive off-springs developed tumor. At age of 10 wk, the mice showed a palpable abdominal mass and became gradually depressed and moribund (Figure 3A). After the skin of abdomen was dissected,

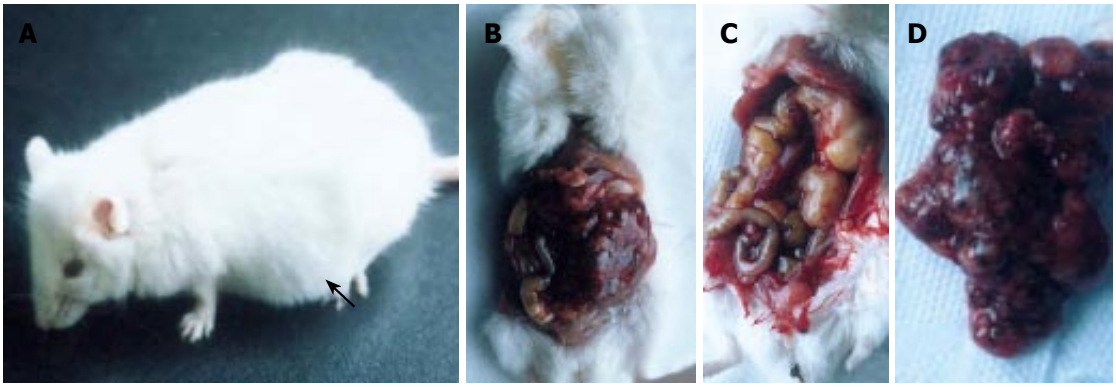


Figure 3 Appearance and anatomy of transgenic mice. **A**: Transgenic mice showing ridgy belly (arrowhead); **B**: Transgenic mice showing a tumor in its abdominal cavity; **C** and **D**: Abdominal cavity and tumor excised from the body.

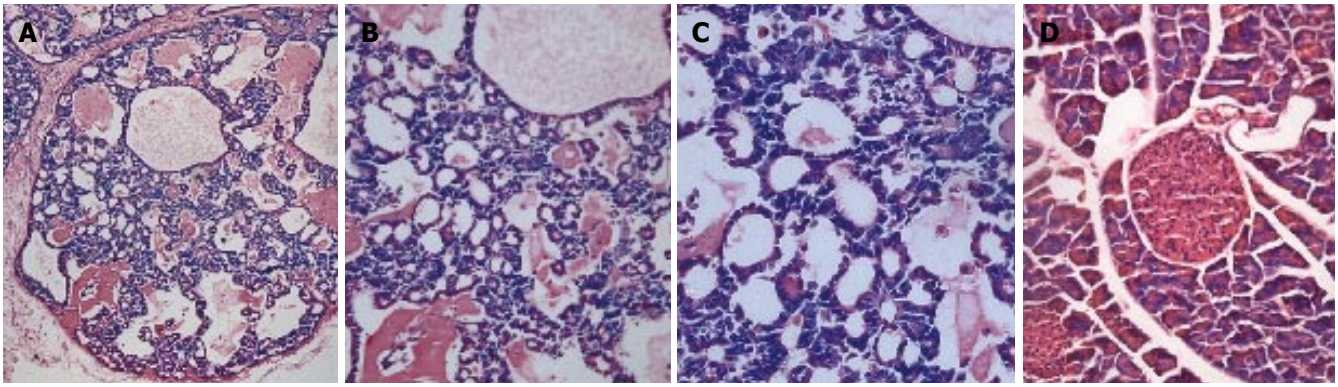


Figure 4 Normal pancreas and tumor tissues-stained with H&E. **A**, **B** and **C**: Tissue of cystic neoplams from transgenic mice (**A**: × 100; **B**: × 200; **C**: × 400); **D**: Normal pancreas with islet and acinar cell (× 200).

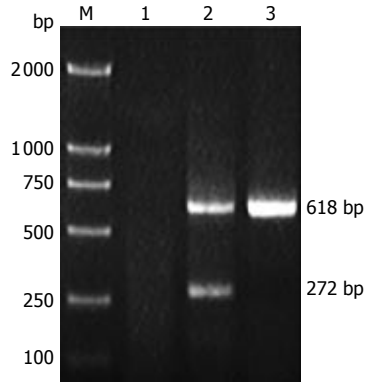


Figure 5 RT-PCR results of transgene expression in tumor. M: DL-2000 marker, 1: blank control (H_2O), 2: cDNA of tumor tissue, 3: negative control (non-reverse transcripted RNA).

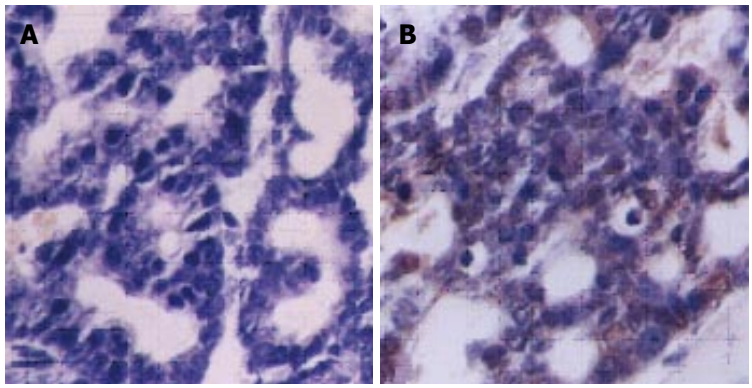


Figure 6 SV40Tag expression in tumor tissue detected by immunohistochemistry without anti-Tag antibody (**A**) and with anti-Tag antibody (**B**).

tumor tissue in the abdominal cavity was observed across peritoneum. In addition, a multilocular cystic tumor was identified in the abdominal cavity (Figures 3B and 3C). The diameter of the cysts ranged from 2 mm to 2 cm. The cystic cavities were filled with wine fluid (Figure 3D). Additionally, the spleen and kidney became swollen, and the kidney showed obvious pathological changes. No other obvious pathological changes were observed in other organs (data not shown). The cystic spaces were lined by multi-layer epithelium cells (Figures 4A-4C).

SV40Tag expression in tumor tissue

To examine whether the transgene was expressed in multiple tissues and tumor of transgenic mice, we performed RT-PCR for SV40Tag. A 272 bp PCR product was generated from tumor cDNA (Figure 5) by using SV40Tag specific primers, but not in other tissues. Furthermore, immunohistochemical analysis detected SV40Tag protein expression in tumor tissues of transgenic mice (Figure 6).

DISCUSSION

The early region of simian virus 40 (SV40) encodes a tumor antigen (Tag), a multifunctional regulatory protein responsible for the alterations of cellular processes is

required for viral infection[7]. SV40 Tag forms complexes with and inactivates the key cell cycle regulatory proteins, such as p53 and retinoblastoma (Rb) tumor suppressor gene product[7-10]. Loss of these functional tumor suppresso gene products is often associated with human cancer. SV40 Tag expression under the control of tissue specific promoters has been used to generate a variety of transgenic models for human cancers[11-14]. We have used breast-specific β-casein promoter for SV40 Tag transgenic models. Since the β-casein promoter can specifically drive the expression of SV40 Tag in the breast, we expected to generate a breast cancer transgenic mice. Surprisingly, the transgenic mice failed to produce breast cancer but developed pancreatic neoplastic lesions. One possible explanation is that the transgene has been integrated into a particular location of the mouse genome and leads to the expression of SV40 Tag in the pancreas. Another possibility is that the regulated element of the expression vector has been activated in the pancreas. In addition, the transgenic female mice developed a pancreatic tumor and died before lactation. Thus we have no way to know whether SV40 Tag is expressed in the galactophore of the transgenic mice.

Cystic pancreatic neoplasms comprise a heterogeneous group of pathologic entities. Although the incidence rate and lethality of the cystic pancreatic neoplasm are not as high as that of the pancreatic adenocarcinoma, some of the cystic pancreatic neoplasms can develop into metastatic pancreatic adenocarcinoma. The most common cystic pancreatic neoplasms are mucinous cystic neoplasm and serous cystadenoma. These two entities account for more than 75% of the cystic pancreatic neoplasms. Other less common cystic pancreatic neoplasms include papillary cystic tumor (also known as solid and papillary tumor), cystic neuroendocrine tumor, acinar cystadenocarcinoma, cystic teratoma, lymphangioma, hemangioma, and paraganglioma[15]. Due to the low incidence and recent nosographic arrangement, the natural evolution and the clinical development of these neoplasms are not well known. It is, therefore, difficult to identify all types of these cystic lesions. Pancreatic cancers are rarely observed spontaneously or induced by carcinogen administration in the laboratory mice. However, genetic engineering has been shown to effectively generate mice with germline oncogenic lesions similar to human pancreatic adenocarcinomas[16]. We have generated a transgenic animal model with classic features of human cystic pancreatic cancer. This model provides a tractable genetic system to dissect the complexities of evolving cancers in a physiological context. We believe that the mouse model is helpful for the identification of early disease markers and effective therapeutic targets for the disease.

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RAPID COMMUNICATION

Analysis of variabilities of serum proteomic spectra in patients with gastric cancer before and after operation

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Abstract

AIM: To study the variabilities of serum proteomic spectra in patients with gastric cancer before and after operation in order to detect the specific protein markers that can be used for quick diagnosis of gastric cancer.

METHODS: Proteomic spectra of 46 serum samples from patients with gastric cancer before and after operation and 40 from normal individuals were generated by IMAC-Cu protein chip and surface-enhanced laser desorption/ ionization time of flight mass spectrometry.

RESULTS: Fourteen differentially expressed proteins in serum were screened by analysis of proteomic spectra of preoperative patients and normal individuals. We obtained 4 proteins (heat shock protein 27, glucose-regulated protein, prohibitin, protein disulfide isomerase A3) making up marker pattern which was able to class the patient-team and normal-team. These marker patterns yielded 95.7% sensitivity and 92.5% specificity, respectively. The proteins over-expressed in serum of preoperative patients were obviously down-regulated.

CONCLUSION: Specific protein markers of gastric cancer can be used for the quick diagnosis of gastric cancer and judgment of prognosis. SELDI-TOF-MS is a useful tool for the detection and identification of new protein markers in serum.

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Key words: Gastric cancer; Proteome; Surface-enhanced laser desorption/ionization time of flight mass spectrometry protein chip technology; Specific marker

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INTRODUCTION

Gastric cancer is a common fast growing malignant tumor and takes the first place of the malignant tumors of digestive canal. About 160 000 deaths of gastric cancer patients occur every year in China[1]. It threatens people' s health in our country. The clinical diagnostic methods of gastric cancer are usually physical and pathologic diagnosis, which can only generally find the patients with intermediate stage. The technologies are complex and the patients wait a final diagnosis for a long time. We can only detect the specific protein markers expressed in gastric cancer by biochemical method to achieve early, quick diagnosis. But so far, no marker is completely specific to gastric cancer. Since the sensitivity and specificity of clinical diagnostic method of gastric cancer are not high, it is urgent to explore and set up an early gastric cancer diagnostic method which is easier, quicker and has a higher sensitivity and specificity.

We utilized surface-enhanced laser desorption/ ionization time of flight mass spectrometry (SELDI-TOF-MS) protein chip technology to contrast and analyze the changes of serum proteomic spectra in patients with gastric cancer before and after operation in order to screen the specific protein markers that could be used for diagnosing gastric cancer and judgment of prognosis.

MATERIALS AND METHODS

General documents

We chose 46 patients with gastric cancer from Department of Oncosurgery of our hospital in 2003 and 2004. Of the 46 patients, 5 were early cases, accounting for 10.9% of the total, and 41 were in intermediate and advanced stage, accounting for 89.1% of the total. The proportion of men and women was 2.2:1.0, the average age was 51 years. Forty healthy people served as the control group, the proportion of men and women was 2.1:1.0, their average age was 49 years. Before and after 15d of operation 5mL axenic venous blood was drawn from each patient on an empty stomach in the early morning and stored at -80 ℃. All the samples were provided by oncosurgery Department

Table 1 Seven over-expressed proteins in gastric cancer

Protein No.	Average molecular weight (kDa)	Protein identified
1	22.33	Heat shock protein 27
2	22.59	Transgelin
3	28.42	Glucose-regulated protein
4	29.90	Prohibitin
5	35.72	NSP3
6	42.46	Unnamed protein product
7	56.77	Protein disulfide isomerase A3

in our hospital. There was not statistical difference in sex proportion and age between the patient and control groups, and there was no relevant disease that could change serum protein content in the two groups.

Materials

Urea, acetonitrile, cibacron blue, 3-gryethylamine-1-propanesulfonic acid (CHAPS), trifluoroacetic acid (TFA), sinapinic acid (SPA) were all bought from Sigma Company. SELDI-TOF-MS, K30 size selection spin column and IMAC3 protein chip were produced by Ciphergen Company in USA.

Serum sample preparation

The samples stored at -80 ℃ were deliquesced at common temperature, cibacron blue and K30 size selection spin column were used to remove albumin in the samples. Then we used IMAC buffer solution (100 mmol/L Na_3PO_4 +250 mmol/L NaCl, pH 7.0) to dilute 20 μL serum sample.

Preparation of IMAC3 protein chip

We installed the IMAC#3 chip onto the bio-processor, added 50 μL of copper sulphate (l00 mmol/L) into every hole, spun it at 4 ℃ with the rotation speed at 200 per minute for 5 min, made copper ion combined with active surface of the chip. The remaining blotted copper sulphate was used to flush the chip in deionized water. Fifty μL of acetic acid sodium (100 mmol/L, pH 4.0) was added and spun to elute un-conjugated copper ion. The liquid was discarded and 150 μL balanced solution (50 mmol/L HEPES, pH 7.0) was added into every hole. Then 50 μL sample was added and incubated at 4 ℃ for 1 h. Excess liquid was discarded and 150 μL HEPES balanced solution flushing 3 times was used to elute the protein or peptide pieces that did not combine with the surface of the chip. Then we used PAP hydrophobic pen to draw the sample hole surrounding the chip and air dried it. After that, we added 0.5 μL SPA solution twice in every hole.

Data reading and result analysis

The prepared chip was analyzed by the analytic equipment of protein chip. Data were obtained by using Ciphergen Proteinchip software. Before the data were obtained, angiotensin peptide was used to correct the equipment to make the mass deviation of system less than 0.1%. The parameters of the data reading were as follows. Laser intensity was 175, detection sensibility was 8, grading-up molecular weight limit was 1500-100 000 Da, best focal setting centre was 6000, parameter limit of data collection was 20-80. Biomarker Wizard software was used to process the obtained data, the proteinic wave crest intensity was calculated, which had the same e/m. Different degrees were indicated by *P* value.

Statistical analysis

Data were analyzed using Biomarker Wizard software and Biomarker Patten software. $P < 0.05$ was considered statistically significant.

RESULTS

Distributed results of serum proteomic spectra between patients with gastric cancer before operation and normal control

Peak detection The protein molecular weight limit detected in the samples from 46 patients with gastric cancer before operation and 40 healthy people was set between 1500 and 100 000 Da. The proteins or peptide pieces less than 1500 Da were precluded, because between 0 and 1500 Da there were many agent compositions, SPA and other chemical compositions which could influence data analysis. The IMAC#3 array was analyzed by the analytic equipment of protein chip. The proteins obtained were expressed as spectrum and 54 126 wave crests were obtained.

Peak alignment and proteomic spectrum analysis Spectra obtained were analyzed by using Biomarker Wizard software. The content of most proteins in samples was generally identical in the preoperative and control groups, but 33 proteins were still differentially expressed in the two groups, 14 proteins of which were significantly differentially expressed in the 33 proteins in the two groups ($P < 0.05$). In these 14 proteins, seven proteins were over-expressed in the 46 patients with gastric cancer before operation, the average molecular weight of them was 22.33, 22.59, 28.42, 29.90, 35.72, 42.46 and 56.77 kDa (named gastric cancer over-expressed serum proteins 1-7), respectively. Compared with control group, seven proteins were under-expressed, the average molecular weights of them were 17.16, 22.02, 30.77, 46.73, 53.40, 77.07 and 80.02 kDa (named gastric cancer under-expressed serum proteins 1-7), respectively. Fourteen proteins were identified, the result showed that the seven over-expressed proteins were heat shock protein (hsp) 27, transgelin, glucose-regulated protein, prohibitin, NSP3, unnamed protein product and protein disulfide isomerase A3 (Table 1). The seven under-expressed proteins were p20, nucleoside diphosphate isomerase A, apolipoprotein A-1, alpha 1 antitrypsin, desmin, serotransferrin and serum albumin (Table 2).

Screening for gastric cancer serum protein markers and establishment of diagnostic pattern The data of the differentially expressed proteins were used to establish a database. Using Biomarker Pattern intellective statistical analysis software to select corresponding conditions, we analyzed the data obtained from preoperative and control groups and obtained the specific proteinic markers of gastric cancer. The results showed that using the pattern

Table 2 Seven under-expressed proteins in gastric cancer

Protein No.	Average molecular weight (kDa)	Protein identified
1	17.16	P20
2	22.02	Nucleoside diphosphate isomerase A
3	30.77	Apolipoprotein A-1
4	46.73	Alpha 1 antitrypsin
5	53.40	Desmin
6	70.07	Serotransferrin
7	80.02	Serum albumin

composed of heat shock protein 27, glucose- regulated protein, prohibitin, protein disulfide isomerase A3 could exactly distinguish gastric cancer patients from healthy people. Using these markers, we established the taxonomic tree pattern of gastric cancer diagnosis, which has two layers and three nodes and could exactly distinguish 95.7% of gastric cancer patients from 92.5% healthy people. The study results showed that these marker patterns yielded 95.7% sensitivity and 92.5% specificity, respectively.

Variabilities of serum proteomic spectra in patients with gastric cancer before and after operation

The sera of patients with gastric cancer after operation were analyzed by using IMAC#3 protein chip and Biomarker Wizard software with the same condition and parameters. The results were compared with those in the preoperative and control groups. The results showed that the expression of heat shock protein 27, glucose-regulated protein, prohibitin, unnamed protein product and protein disulfide isomerase A3 (namely: gastric cancer over-expressed serum proteins 1, 3, 4, 6, 7) decreased obviously in postoperative group compared with control group. The five proteins had significantly different expression. When compared with the preoperative group, the five proteins had significantly different expression ($P < 0.05$). NSP3 and transgelin (namely: gastric cancer over- expressed serum proteins 2, 5) were not obviously down-regulated after operation and still over-expressed. When compared with the preoperative group, they were not significantly expressed. When compared with control group, they were significantly expressed ($P < 0.05$) (Table 3). Compared with the preoperative and control groups, the results of serum proteomic spectra in postoperative group showed that serum proteomic spectra had some variabilities and the expression of some proteins with heavier molecular weight was different from that in the above two groups.

Table 3 Variabilities of gastric cancer serum over-expressed proteins before and after operation

Proteins	Molecular weight (kDa)	Expression preoperation	Expression postoperation
Heat shock protein 27	22.33	Over-expressed[a]	Normal[c]
Transgelin	22.59	Over-expressed[a]	Over-expressed[a]
Glucose- regulated protein	28.42	Over-expressed[a]	Normal[c]
Prohibitin	29.90	Over-expressed[a]	Normal[c]
NSP3	35.72	Over-expressed[a]	Over-expressed[a]
Unnamed protein product	42.46	Over-expressed[a]	Normal[c]
Protein disulfide isomerase A3	56.77	Over-expressed[a]	Normal[c]

[a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* preoperative group.

DISCUSSION

Gastric cancer is a frequently encountered malignant tumor in clinic. Gastric cancer possesses a natural bacterial drug resistance to many chemotherapeutics. The current situations of gastric cancer in our country are high case fatality, low early diagnosis rate, low exairesis rate and low five-year survival rate. The method to diagnose gastric cancer is very complex. Pathogenesis of gastric cancer is a long process. Multiple factors determine genetic predisposition, which refers to bacterial infection, abnormal changes of many oncogenes and anti-oncogenes, including activation of oncogene, inactivation or absence of anti-oncogene, genic mutation, etc. It was reported that infection with *helicobacter pylori* could result in gastric epithelial hyperplasia and apoptosis[2]. Decrease of folacin in cells reduces the methylated level of some DNA fragments, including certain oncogenes[3]. Some scholars believe that the lower the methylated level of genes is the poorer the differentiation of the cancer cell[4]. The deletion of nm23 gene may relate with the transfer process of gastric cancer. It is necessary to find a new, effective and quick diagnostic technique which has a high sensibility and specificity to diagnose gastric cancer.

Before pathological change is discovered, the component and quantity of intracellular proteins may have corresponding changes in the disease. Through dynamic observation of proteins, pristine disease signs can be discovered. Proteomic technology has made the screening possible and can establish a new molecular diagnostic technology for clinical disease diagnosis, especially for tumor diagnosis[5]. Proteomic studies could lead to molecular characterization of cellular events associated with cancer progression, signaling and developmental stages[6,7]. Cancer-specific protein markers are the basis for developing new methods for early diagnosis of gastric cancer and its progression[7-10]. Genes work at protein level. Since the proteomic results can express both the intrinsic genetic effect on cells and the impact of its environment, it is very valuable to determine biomarkers of tumor. In the past, the main technologies used in protein research are 2D electrophoresis (2DE), high performance liquid chromatography (HPLC), mass spectrum, etc. Because information obtained is not enough, the development of proteomics is confined[11,12]. SELDI-TOF-MS protein chip technology is a new proteomic technology and can be used in high-flux analysis. Because the protein chip system based on this technology is widely used to screen early diagnostic markers of tumor, significant results have been achieved. Eggeling *et al*[13] have obtained the proteomic spectral variation of renal cancer by using this technology. Paweletz *et al*[14] investigated the proteomic spectral variation of liquid drawn from nipple and showed that

the proteomic spectra in patients with mammary cancer are significantly different from those in healthy people. Vlahou *et al*[15] have screened out five specific markers of bladder cancer from urine of patients with bladder cancer. These findings indicate that SELDI-TOF-MS protein chip technology is one of the main technologies of proteomics, especially suitable for screening the specific markers of tumor.

The results of our study showed that 33 proteins were differentially expressed in preoperative and control groups, 14 proteins of which were significantly expressed ($P<0.05$). In these 14 proteins, seven proteins were over-expressed and seven proteins were under-expressed. Through the identification of the 14 proteins, the seven over-expressed proteins were heat shock protein 27, transgelin, glucose-regulated protein, prohibitin, NSP3, unnamed protein product and protein disulfide isomerase A3, while the seven under-expressed proteins were p20, nucleoside diphosphate isomerase A, apolipoprotein A-1, alpha 1 antitrypsin, desmin, serotransferrin and serum albumin. The data of differentially expressed proteins were used to establish the database. The Biomarker Pattern intellective statistical analysis software was used to analyze the data in the database. We established a diagnostic pattern composed of heat shock protein 27, glucose-regulated protein, prohibitin, protein disulfide isomerase A3, which can exactly distinguish gastric cancer patients from healthy people. If these markers are used to diagnose early gastric cancer, they are able to yield a 95.7% sensitivity and a 92.5% specificity, respectively.

The serum proteomic spectra also changed in patients with gastric cancer after operation. The five formerly over-expressed proteins, namely heat shock protein 27, glucose-regulated protein, prohibitin, unnamed protein product and protein disulfide isomerase A3, were obviously down-regulated. When compared with control group, the five proteins were not significantly expressed ($P>0.05$), but the two proteins, namely transgelin and NSP3, were not obviously down-regulated. When compared with control group, the two proteins were over-expressed ($P<0.05$). The results of our study suggest that using the diagnostic pattern composed of the above four proteins to diagnose gastric cancer, can obtain a high sensibility and specificity and it can be widely used for clinical diagnosis of gastric cancer. But because the cases in early stage accounted for a small proportion of the samples in our research, the technology can only be used to diagnose gastric cancer. Before we give a conclusion whether the technology can be used to screen early gastric cancer, we must collect more cases of early gastric cancer.

In a word, using the diagnostic pattern composed of heat shock protein 27, glucose-regulated protein, prohibitin, protein disulfide isomerase A3 to diagnose gastric cancer can obtain higher positive rate, higher sensibility and specificity. It can be used to diagnose gastric cancer quickly and exactly.

In conclusion, SELDI-TOF-MS protein chip technology has lots of advantages and is a new effective technology for study of proteins.

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CASE REPORT

Long-term survival of a case with multiple liver metastases from duodenal gastrointestinal stromal tumor drastically reduced by the treatment with imatinib and hepatectomy

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Abstract

Constitutive activation of KIT receptor tyrosine kinase is a critical factor in the pathogenesis of gastrointestinal stromal tumors (GISTs). But there is little information on whether combination of imatinib mesylate (IM) and surgical treatment can prolong survival in the cases with unresectable multiple liver metastases. We report a case of postoperative recurrence of GIST treated by the tyrosine kinase inhibitor IM and surgical treatment. The initial complete response (CR) to treatment continued for 18 mo, but single liver metastasis showed regrowth in the left hepatic lobe during IM treatment. After partial resection of the recurrent tumor, postoperative course was uneventful and the patient has survived without recurrence for 24 mo. Currently, imatinib is the first-line therapy for non-resectable GISTs, but a single agent therapy often leads to tumor resistance. Even if tolerance to imatinib occurs, a combination of imatinib and surgical treatment can prolong survival in some cases as reported here. However, further studies on a large number of cases of recurrent GIST are necessary to evaluate the effectiveness of IM treatment combined with surgery.

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Key words: GIST; Imatinib mesylate; Liver metastases

Sakakura C, Hagiwara A, Soga K, Miyagawa K, Nakashima S, Yoshikawa T, Kin S, Nakase Y, Yamaoka N, Sagara Y, Yamagishi H. Long-term survival of a case with multiple liver metastases from duodenal gastrointestinal stromal tumor drastically reduced by the treatment with imatinib and hepatectomy. *World J Gastroenterol* 2006; 12(17): 2793-2797

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are a group of mesenchymal neoplasms that arise from Cajal cells which are pacemaker cells of the gastrointestinal tract[1]. These tumors occur predominantly in middle-aged and older persons, and approximately 70% of the tumors are found in the stomach, 20%-30% are found in the small intestine, and less than 10% elsewhere in the gastrointestinal tract[1,2]. Recent studies have shown that cells GISTs express a growth factor receptor with tyrosine kinase activity termed c-kit[2]. This gene product appears to be the most specific diagnostic criterion for the diagnosis of GISTs[3]. The ligand for the c-kit receptor is the stem-cell factor, also known as the steel factor or c-kit ligand[4]. Mutations of c-kit that cause constitutive activation of the tyrosine kinase function of c-kit are detectable in most GISTs and appear to play a central part in their pathogenesis[2,5]. These mutations result in ligand-independent tyrosine kinase activity, autophosphorylation of c-kit, uncontrolled cell proliferation, and stimulation of downstream signaling pathways, including those involving phosphatidylinositol 3-kinase and mitogen-activated protein kinases. GISTs are notoriously unresponsive to conventional chemotherapy, and there is no effective therapy for the advanced, metastatic stage of this disease[6]. In this study, we used imatinib (STI571 or Glivec, Novartis, Basel, Switzerland)[7], an inhibitor of the tyrosine kinase activity of c-kit, for a patient with a GIST with multiple liver metastases.

CASE REPORT

In January 2003, a 37-year-old, previously healthy man, presented with mild abdominal discomfort and a large mass in the upper abdomen. Examination with upper gastrointestinal tract led to removal of the tumor in the

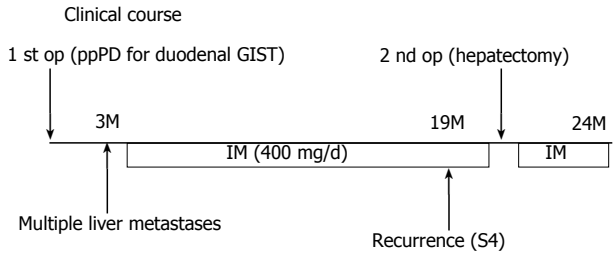


Figure 1 Clinical course of the patient illustrating the operations (pancreaticoduodenectomy for duodenal GIST and partial resection of recurrence tumor) and IM treatment.

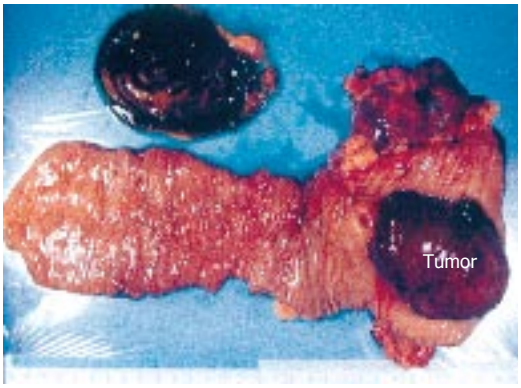


Figure 2 Surgical specimen obtained by pancreaticoduodenectomy of the duodenal GIST.

second portion of the duodenum by means of pancreaticoduodenectomy (PD). Clinical course of the operations (pancreaticoduodenectomy for the duodenal GIST and partial resection of recurrence tumor) and IM treatment are illustrated in Figure 1. The resected specimen is shown in Figure 2. In the second portion of the duodenum, a tumor of 5 cm in diameter was observed. Immunohistochemistry showed the tumor cells to be c-kit-positive, CD34-positive, SMA-negative, and S100-negative (Figure 3), indicating the tumor as a GIST. Histologic examination of the specimens revealed more than 20 cells per 10 high-power fields undergoing mitosis and identified the masses as a GIST. The diagnosis was confirmed by immunostaining for CD117, and c-kit mutation consisting of point mutation in exon 11 was detected in tumor DNA amplified by polymerase chain reaction[8]. Three months later, in April 2003, multiple recurrent tumors in the liver were detected by magnetic resonance imaging (MRI) and PET (Figures 4 and 5).

The patient was then treated with imatinib after his written informed consent had been obtained. Treatment with four 100 mg capsules of imatinib once daily was started in May 2003. This dose was based on evaluations of the safety and tolerability of imatinib for patients with chronic myeloid leukemia[9]. Toxicity was assessed at follow-up visits every two to four weeks, and blood cell counts and blood chemistry were examined every one to two weeks. The response to treatment was assessed with dynamic MRI and PET with ^{18}F-fluorodeoxyglucose as a tracer.

When measured as the sum of the products of the two

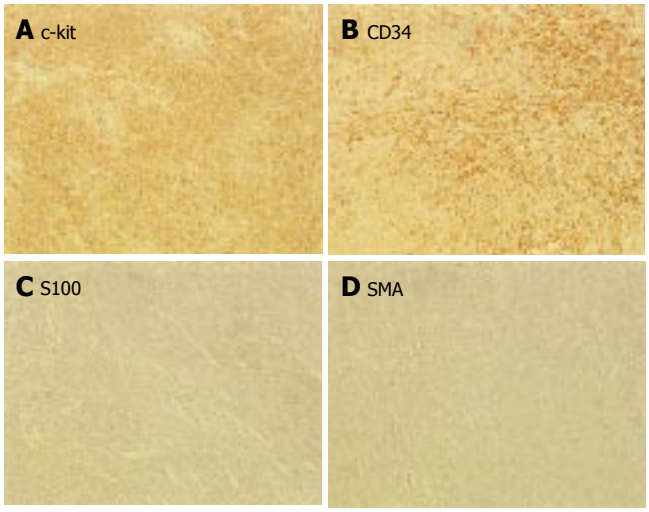


Figure 3 Immunohistochemical analysis of the duodenal GIST. **A:** c-kit; **B:** CD34; **C:** S100; **D:** SMA.

perpendicular axes of each of six large liver metastases, the size of the tumor one day before the start of imatinib treatment was 42 cm^2, but subsequent MRI scans showed that the size of the tumor had been reduced to 1 cm^2. No enhancement was seen on dynamic MRI obtained during the treatment, and many of the metastases became hypodense. Until October 2004, the tumors at all sites had continued to respond to the treatment, and the patient had remained clinically well. Multiple liver metastases and increased accumulation of ^{18}F-fluorodeoxyglucose were observed on the first PET obtained 4 d before the treatment with imatinib was started. One month after the treatment, PET scan showed no abnormal uptake of ^{18}F-fluorodeoxyglucose in the liver. On the PET scan obtained after two months of the imatinib treatment, cold areas with less uptake of ^{18}F-fluorodeoxyglucose than in the surrounding liver parenchyma were seen at the sites of liver metastases.

Although the response to treatment was initially CR and continued for 16 mo, single liver metastasis showed regrowth in the left lobe of the liver (S4) during the imatinib treatment. Other sites remained hypodense and no sign of recurrence was seen (Figure 6). Partial resection of recurrent tumor resulted a satisfactory postoperative course and the patient remains still alive without recurrence for 24 mo since the initial treatment with IM. The effect of IM treatment on other liver metastases besides the lesion of left hepatic lobe continued (Figures 6 and 7).

Mutation analysis of c-kit in exons 7, 11, and 13 was performed in the primary tumor as well as recurrent tumor as previously described[9]. Primary tumor showed the c-kit mutation at exon 11 codon 567-576 (AAT TAT GTT TAC ATA GAC CCA ACA CAA CTT → GAA), and an additional mutation was observed at exon 17 codon 820 GAT(Asp) to GGT(Gly) in recurrent tumor in the liver.

DISCUSSION

There is no effective therapy for unresectable or metastastic GIST, which is invariably fatal. The management of

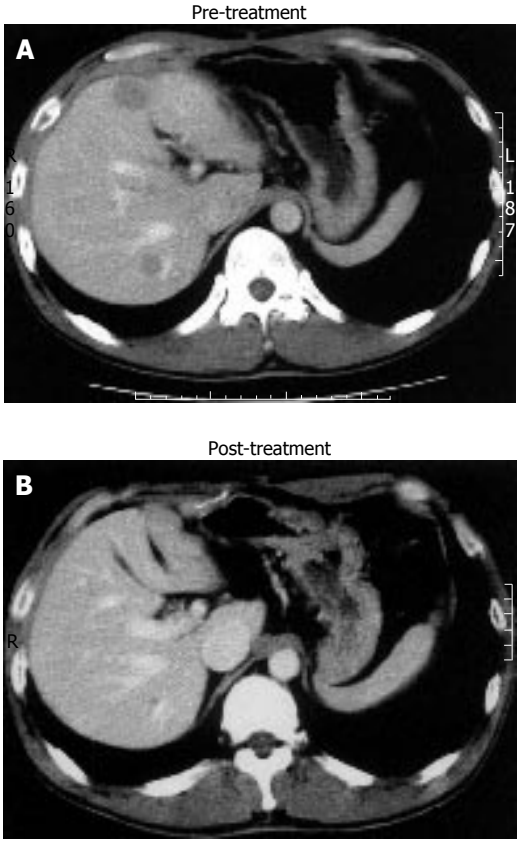


Figure 4 Abdominal MRI showing multiple liver metastases and complete remission in response to imatinib therapy. Panel **A:** MRI images of the patient with multiple liver metastases after 3 mo of pancreaticoduodenectomy for malignant duodenal GIST; Panel **B:** Follow-up MRI images after 1 mo of oral imatinib therapy at 400 mg daily.

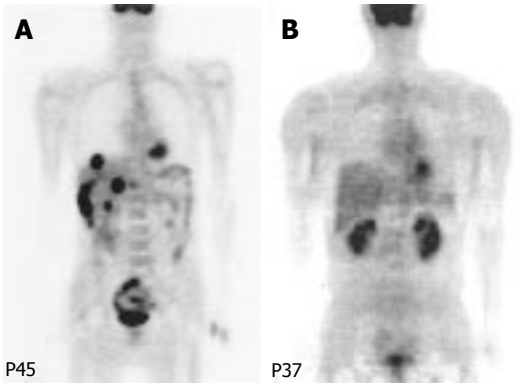


Figure 5 PET scan of liver metastases before and after imatinib treatment. **A:** pretreatment with imatinib; **B:** posttreatment with imatinib.

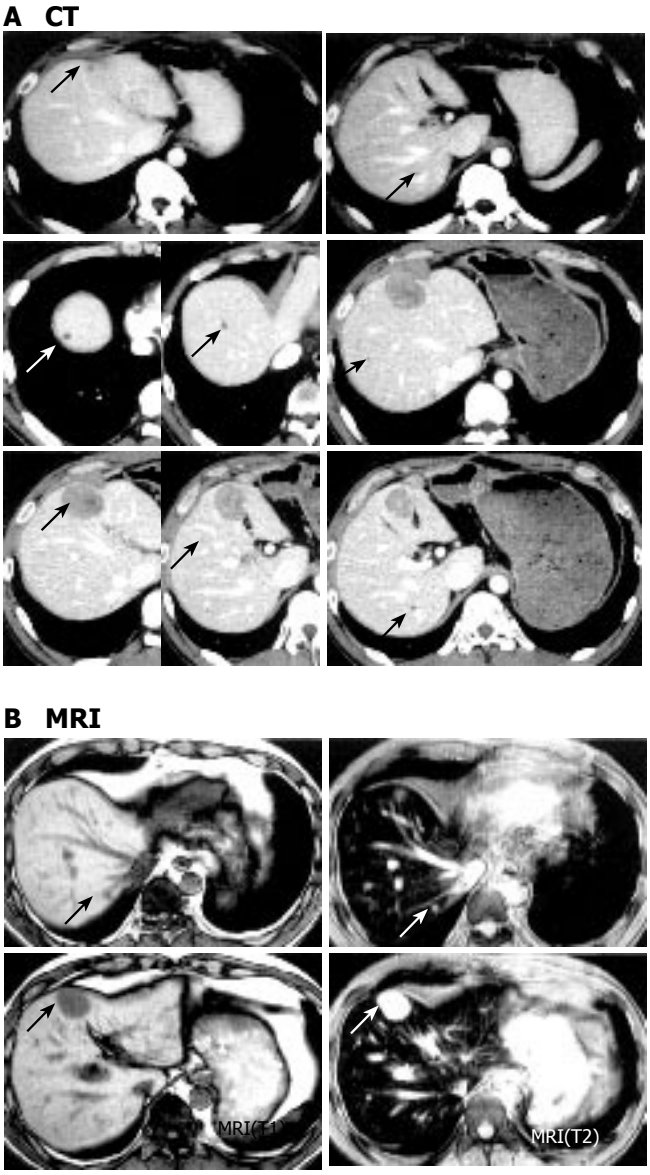


Figure 6 Abdominal CT and MRI showing recurrence of liver metastasis. **A:** CT; **B:** MRI.

unresectable or metastatic GISTs has been difficult because they are resistant to conventional chemotherapy and radiation.

Imatinib, a phenylaminopyrimidine derivative, is a small molecule that selectively inhibits the enzymatic activity of several tyrosine kinases, including ABL and the BCR-ABL fusion protein of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. This selective activity of imatinib suggests that its inhibition of the constitutively active mutant c-kit tyrosine kinase seen in GIST can be an effective therapy for these tumors. Our patient had a rapidly progressive metastatic GIST. He showed a complete metabolic response within one month after the start of imatinib treatment, as shown by negative findings on PET, which indicated reduced tumor viability. In this case, histopathological evaluation and serial MRI analysis could not be performed soon, but dramatic shrinkage of the metastastic liver tumors was observed within two months. The close relation between clinical outcome and the findings on ^{18}F-fluoro-2-deoxy-D-glucose PET scanning indicates that such scanning is a useful complement to standard anatomical imaging with CT or MRI for monitoring the therapeutic effect of imatinib in patients with GISTs. Side effects of imatinib include nausea, diarrhea, edema, leukocytopenia, GI tract bleeding, liver dysfunction and so on[10]. In our case, however, only mild liver dysfunction was observed, and treatment with imatinib could be continued safely. This effect continued for more than 16 mo and there has

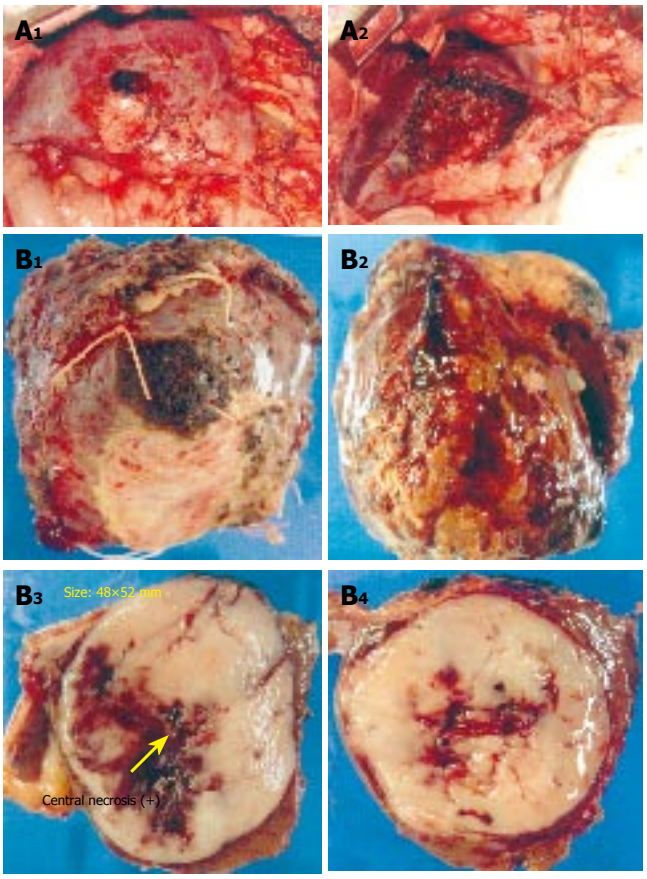


Figure 7 Surgical specimen of partial hepatectomy for GIST recurrence. **A:** Operative findings; **B:** Resected specimens.

been no sign of the recurrence. However, recurrence was subsequently detected and partial resection of recurrent tumor was performed.

It has been reported that the initial phase II study of imatinib treatment for metastatic GIST reported long-lasting responses in patients who followed up for 13 mo. However, it has become evident with extended follow-up that tumor progression can occur despite initially positive responses to imatinib therapy. The median duration of the responses was 19 mo for a series of patients with 2-year progression-free survival rate of 38%, which is a substantial drop from 90% at a 1 year[11]. Similar to previously reported cases, our case showed recurrence 16 mo after initial treatment with IM in one of six metastatic lesions. Resectability was confirmed and hepatic resection of the recurrence was successfully performed. The combination of IM and surgical treatment can prolong survival as shown in our case. Therefore, if the recurrent tumor is resectable, a second operation combined with adjuvant therapy with IM should be performed. If tumor re-growth occurs, surgical treatment should thus be considered.

Several studies have reported the relationship between c-kit mutation and imatinib sensitivity[11-19]. Although the mechanism of tolerance to imatinib is still unclear, screening of kit mutation may be useful for prediction of the imatinib sensitivity and recurrence. Many kinds of c-kit mutations related to IM resistance have been reported[11,13-19]. In our case, a mutation in exon 11 of KIT was initially noted. After interruption of the treatment, an additional point mutation arose in exon 17 that caused resistance to imanitib. It has been postulated that the emergence of c-kit mutations that are resistant to imatinib-targeted therapy may account for these late treatment failures, and investigation into the biologic phenomena of imatinib resistance is an area of current research. As the mechanisms of recurrence and resistance to imatinib in GIST remains unclear, they should be investigated in detail from both the clinical and molecular biological point of view.

Imatinib is a recent and very promising treatment modality for the GIST, but complete surgical extirpation remains the only curative treatment of malignant GIST, as evidenced by our patient, who became disease-free only after resection. Even then, however, significant risk of recurrence remains, despite successful resection and imatinib treatment. Our patient's favorable response to imatinib supports the concept that specific inhibition of tyrosine kinase is a clinically useful therapeutic intervention for tumors in which aberrant kinase signaling is critical. Depending on individual circumstances, we should choose the most desirable treatment modality, and the combination of surgical extirpation and imatinib therapy should help to improve the prognosis of GIST patients in some cases. Further studies are necessary to clarify the various mechanisms involved.

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CASE REPORT

Post-traumatic hepatic artery pseudo-aneurysm combined with subphrenic liver abscess treated with embolization

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Abstract

A 23-year-old man with post-traumatic hepatic artery pseudo-aneurysm and subphrenic liver abscess was admitted. He underwent coil embolization of hepatic artery pseudo-aneurysm. The pseudo-aneurysm was successfully obstructed and subphrenic liver abscess was controlled. Super-selective trans-catheter coil embolization may represent an effective treatment for hepatic artery pseudo-aneurysm combined with subphrenic liver abscess in the absence of other therapeutic alternatives.

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Key words: Pseudo-aneurysm; Hepatic trauma; Liver abscess

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INTRODUCTION

Hepatic artery pseudo-aneurysm is a rare complication of blunt abdominal trauma. Diagnosis is frequently delayed and made by splanchnic angiography. Most of the indications for surgical treatment may disappear after selective catheterization and embolization. We report a case of a 23-year-old man with a history of hepatic trauma. Subphrenic abscess formation after laparotomy and right hepatic artery pseudoaneurysm were diagnosed by enhanced computer tomography (CT). The lesion was confirmed by arteriography and managed with trans-catheter embolization.

CASE REPORT

We report a case of a 23-year-old man with a severe blunt abdominal trauma caused by a car accident. An immediate laparotomy was performed due to a severe collapse upon admission and a liver fracture was observed. He gradually recovered and a subphrenic abscess occurred on day 10 after operation. Abdominal CT scanning revealed a complex subphrenic abscess and a pseudo-aneurysm of the right hepatic artery (Figure 1). Selective angiography confirmed the diagnosis of right artery pseudo-aneurysm (Figure 2) and the aneurysm was occluded by trans-catheter embolization. This was accomplished uneventfully with placement of two 5mm coils into the aneurysm and right hepatic artery was unblocked (Figure 3). The 14-F drainage catheters containing semi-solid necrotic material were placed into these collections. Eventually, recovery was complete. The classical embolization could avoid surgical treatment with a high morbidity.

DISCUSSION

The liver is the largest solid abdominal organ with a relatively fixed position, which makes it prone to injury. The most common cause of liver injury is blunt abdominal trauma, secondary to motor vehicle accidents in most instances. In the past, most of these injuries were treated surgically. However, surgical literature indicates that as high as 86% of liver injuries have stopped bleeding at the time when surgical exploration is performed, and 67% of operations performed for blunt abdominal trauma are non-therapeutic[1-2].

As a result of this high mortality rate, emergency surgery is frequently indicated in patients with hepatic injury in the past. However, with better monitoring facilities and imaging techniques, most patients with blunt abdominal trauma are now treated conservatively. Pseudoaneurysms are better depicted by spiral or multi-section CT because of the ability to image during peak contrast enhancement[3].

Complications of liver trauma occur in approximately 20% of patients, including delayed rupture (very rare), hemobilia, arterio-venous fistula, pseudo-aneurysm, biloma and abscess formation[4]. There are many potential causes of liver abscesses, such as appendicitis, diverticulitis, or



Figure 1 Axial contrast-enhanced CT scan revealing a vascular mass lesion in the right lobe of liver.

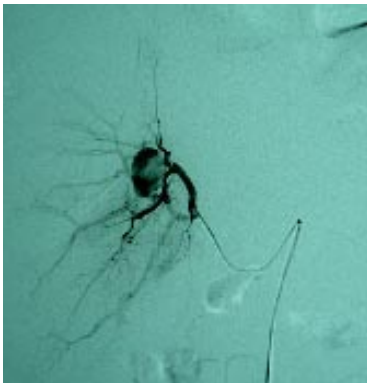


Figure 2 Selective celiac arteriogram showing a hepatic artery pseudo-aneurysm in the right hepatic artery.

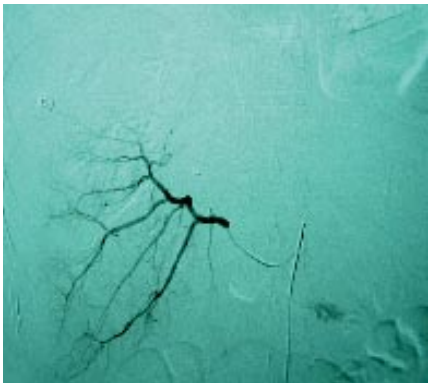


Figure 3 Post embolization selective arteriogram showing disappearance of the right hepatic artery pseudoaneurysm in the right lobe of liver.

a perforated bowel, infection in blood, infection of the biliary (liver secretion) tract, or trauma with bruised liver infection[5].

The most common bacteria that cause liver abscesses are escherichia coli, klebsiella, enterococcus, and bacteroides. The death rate is 10%-30% in treated patients, and higher in those with multiple abscesses. Life-threatening sepsis can develop. The treatment usually consists of surgical or percutaneous (through the skin, with a needle) drainage of the abscess. This is accompanied with a prolonged antibiotic therapy. Sometimes antibiotics alone can cure the infection. Occasionally, patients with blunt abdominal trauma do well initially, but subsequently develop a liver abscess, presumably due to unrecognized liver damage. These patients have signs and symptoms of deep-seated infection[6-7].

A dynamic angiographic study may demonstrate the site of active bleeding and the sac of pseudoaneurysms. Trans-catheter embolization may be the only treatment required. Superselective catheterization should be performed as far as possible. Both afferent and efferent vessels of pseudo-aneurysms or aneurysms should be occluded to prevent their retrograde filling[8]. Initially, the surgeon may control the hemorrhage by temporary perihepatic packing. Recurrent liver parenchymal bleeding can be treated successfully with trans-catheter embolization, and placement of an intravenous stent can control bleeding from a major hepatic vein[9].

Embolization can be performed for the control of persistent arterial hemorrhage which may occur due to stab wounds of the liver, and for the occlusion of pseudo-aneurysm. Trans-catheter arterial embolization may reduce transfusion requirements and allow healing of hepatic injuries without surgery. Because hepatic arteries are not the end arteries, occlusive devices should be deployed distal to the lesion to prevent collateral backdoor filling. The entire hepatic artery may be occluded if required, as long as the portal vein is patent. If the portal vein is occluded, only selective embolization can be performed to prevent liver infarction due to the presence of intrahepatic collaterals[10].

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology
Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
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Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
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www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
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www.czech-hepatology.cz/phm2006

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Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22–25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19–23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22–24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24–27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4–8 February 2006
Titisee
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European Multidisciplinary Colorectal Cancer Congress 2006
12–14 February 2006
Berlin
Congresscare
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www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
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Falk Foundation e.V.
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
Falk Foundation e.V.
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14th United European Gastroenterology Week
21–25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25–28 October 2006
Berlin
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www.codhy.com

Asia Pacific Obesity Conclave
1–5 March 2006
New Delhi
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ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

XXX Panamerican Congress of Gastroenterology
11–16 November 2006
Cancun
www.panamericano2006.org.mx

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
Amsterdam
Continuing Education Inc
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6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
Office of Continuing Medical Education
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Hepatitis 2006
25 February 2006–5 March 2006
Dakar
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mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24–26 February 2006
Sharjah
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www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
Edinburgh
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13th International Symposium on Pancreatic & Biliary Endoscopy
20–23 January 2006
Los Angeles - CA
laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26–28 January 2006
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Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
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American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
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71st ACG Annual Scientific and Postgraduate Course
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The American College of Gastroenterology

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27–31 October 2006
Boston, MA
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3 **Lin GZ,** Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 285-287

Books and other monographs (list all authors)

4 **Sherlock S,** Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

5 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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6 **Morse SS.** Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1):24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
5	2 hours	2 h	After Arabic numerals
6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
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11	0,1,2 years	0, 1, 2 yr	In figures and tables
12	0,1,2 year	0, 1, 2 yr	In figures and tables
13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
15	2 months	2 mo	In figures and tables
16	Two mo	Two months	At the beginning of a sentence
17	10 minutes	10 min	
18	Ten min	Ten minutes	At the beginning of a sentence
19	50% (*V/V*)	500 mL/L	
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21	1 M	1 mol/L	
22	10 μM	10 μmol/L	
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28	25 $g.kg^{-1}/d^{-1}$	25 g/kg per day	
29	96000	96 000	
30	1000 rpm	1000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	1 pg/L	
33	10 kilograms	10 kg	
34	Gene bank	GenBank	International classified genetic materials collection bank
35	Ten L	Ten liters	At the beginning of a sentence
36	Ten mL	Ten milliliters	At the beginning of a sentence
37	umol	μmol	
38	30 sec	30 s	
39	1 g/dl	10 g/L	10-fold conversion
40	OD_{260}	A_{260}	"OD" has been abandoned
41	One g/L	One microgram per liter	At the beginning of a sentence
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43	^bP<0.05	^aP < 0.05	In Table, no note is needed if there is no significance instatistics: ^aP<0.05, ^bP<0.01 (no note if P>0.05). If there is a second set of P value in the same table, ^cP<0.05 and ^dP<0.01 are used for a third set: ^eP<0.05, ^fP<0.01.
44	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F = 9.87, ^2F = 25.9 ^3F = 67.4	Notices in or under a table
45	KM	km	kilometer
46	CM	cm	centimeter
47	MM	mm	millimeter
48	Kg, KG	kg	kilogram
49	Gm, gr	g	gram
50	nt	N	newton
51	l	L	liter
52	db	dB	decibel
53	rpm	r/min	rotation per minute
54	bq	Bq	becquerel, a unit symbol
55	amp	A	ampere
56	coul	C	coulomb
57	HZ	Hz	
58	w	W	watt
59	kPa	mmHg	
60	p	Pa	pascal
61	ev	EV	volt (electronic unit)
62	Jonle	J	joule
63	J/mmol	kJ/mol	kilojoule per mole
64	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
65	N·km	KN·m	moment
66	*x*±s	mean ± SD	In figures, tables or text narration
67	Mean±SEM	mean ± SE	In figures, tables or text narration
68	*im*	im	intramuscular injection
69	*iv*	iv	intravenous injection
70	Wang et al	Wang *et al*	
71	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing
72	Ecoli	*E coli*	Bacteria and other biologic terms have their specific expression
73	Hp, *Helicobacter pylori*	*H pylori*	
74	Iga	*Iga*	writing form of genes
75	*igA*	IgA	writing form of proteins
76	5 mg/kg/h	5 mg/kg per hour	
77	0-29%	0%-29%	
78	Number, No, No. cases, number of mice, number of cases	*n*	In figures and tables

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Hans Gregersen, MD, Asbjorn Mohr Drewes, MD, *Series Editor*

Perception of stimuli in the gastrointestinal physiology and new methods: From the editor

This special issue of *World Journal of Gastroenterology* contains a number of reviews on physiology and perception of stimuli in the gastrointestinal tract. Pain is probably the most prevalent symptom in medicine and characterization of pain is of major importance in the diagnosis and choice of treatment. Consequently, understanding and characterization of the physiology behind gut pain is one of the most important issues in the diagnosis and assessment of organ dysfunction. Research leading to a better insight into pain mechanisms in the gastrointestinal tract will invariably improve the treatment of the patients. Experimental pain models have been used frequently in animal studies. However, animal experiments can only to some degree reflect the experience of clinical pain in humans. Because of these factors, experimental human pain models are advantageous to assess basic pain mechanisms and to investigate abnormal pain responses in functional and organic diseases of the GI system. Using these models, the investigator can control the experimentally induced pain and provide quantitative measures of the responses. Recently, techniques to assess the gastrointestinal anatomy and function have improved dramatically. Imaging with ultrasound combined with endoscope, functional luminal imaging and advanced modeling of the luminal structures with assessment of the mechanical properties of the tissues may be a new cornerstone in experimental and clinical gastroenterology. The key to the progress in these developments have been the interdisciplinary collaboration between doctors, biomedical engineers and mathematicians. In the future the new methods will undoubtedly lead us into a new area bridging the understanding of basic physiology, symptom assessment and daily clinical practice. The reader is referred to the papers in this issue of WJG for information on these important aspects of gastrointestinal function.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, Asbjorn Mohr Drewes, MD, *Series Editor*

Experimental human pain models in gastro-esophageal reflux disease and unexplained chest pain

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Abstract

Methods related to experimental human pain research aim at activating different nociceptors, evoke pain from different organs and activate specific pathways and mechanisms. The different possibilities for using mechanical, electrical, thermal and chemical methods in visceral pain research are discussed with emphasis of combinations (e.g., the multimodal approach). The methods have been used widely in assessment of pain mechanisms in the esophagus and have contributed to our understanding of the symptoms reported in these patients. Hence abnormal activation and plastic changes of central pain pathways seem to play a major role in the symptoms in some patients with gastro-esophageal reflux disease and in patients with functional chest pain of esophageal origin. These findings may lead to an alternative approach for treatment in patients that does not respond to conventional medical or surgical therapy.

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Key words: Pain; Gut; Experimental; Chest pain; Esophagitis

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INTRODUCTION

Pain is probably the most prevalent symptom in medicine and characterization of pain is of major importance in the diagnosis and choice of treatment[1]. Abdominal pain is frequently occurring, even in the normal population[2], and abdominal discomfort and pain are among the most common symptoms responsible for patients consulting the health care system[3]. Consequently, understanding and characterization of gut pain is an important issue in the diagnosis and assessment of organ dysfunction. Research leading to a better insight into pain mechanisms in the gastrointestinal (GI) tract will invariably improve the treatment of the patients[1]. However, in practice, assessment of clinical pain is difficult as the symptoms of the underlying diseases confound the description of pain. These confounders may include complaints relating to psychological, cognitive and social aspects of the illness, as well as systemic reactions such as fever and general malaise[4]. Treatment with analgesics may also cause sedation and other side effects. Finally, abdominal pain is much more diffuse than somatic pain with autonomic symptoms, referred pain and hyperalgesia in remote somatic tissues. These factors will invariable bias the clinical evaluation. For example in the evaluation of analgesics the patients often tend to interpret other effects of the medication-such as an effect on the anxiety and depression relating to the disease-as a relief of pain[5]. To encompass these bias experimental pain models are increasingly used, especially in animal studies. In these experiments, the neuronal nociceptive activity can be recorded or behavior can be assessed[6]. However, neuronal recordings or reactions do not reveal all aspects of pain being the net effect of complex multidimensional mechanisms that involve most parts of the central nervous system[7]. Furthermore, major species differences in pain mechanisms often influence the findings. Therefore, although nociceptive reflexes or electrophysiological recordings from selected pathways in the animal nervous system are important in basic research, the central pain mechanisms and associated complex reactions are typically suppressed. Thus, animal experiments can only to some degree reflect the experience of clinical pain in humans. Because of these factors, experimental human pain models are advantageous to assess basic pain mechanisms and to investigate abnormal pain responses in functional and organic diseases of the GI system. Methods related to

experimental human pain research aim to activate different nociceptors, evoke pain from different organs and activate specific pathways and mechanisms. Assessment of the output from these pain models can be based on psychophysical or neurophysiologic methods[8]. Using these models, the investigator can control the experimentally induced pain (including the nature, localization, intensity, frequency and duration of the stimulus), and provide quantitative measures of the psychophysical, behavioral, or neurophysiologic responses. Methods for imaging can also be added and some studies used a combination of these assessments. In this paper, however, the focus will be the methods for pain induction. The reader is referred to[4] for further detail regarding assessment of GI pain

EXPERIMENTAL PAIN MODELS

The ultimate goal of advanced human experimental pain research is to obtain a better understanding of the mechanisms involved in pain transduction, transmission, and perception under normal and pathophysiological conditions. Experimental approaches can be applied in the laboratory for basic studies and in the clinic to characterize patients with sensory dysfunction and/or pain in organic and functional diseases[4,9]. Depending on the experimental model, different central mechanisms and conditions mimicking pathological pain such as increased sensation to normal physiologic/non-painful and painful stimuli (allodynia and hyperalgesia) can be studied. However, most previous studies have relied on relatively simple mechanical or electrical stimuli. These methods are easy to apply but as discussed below they have special considerations and limitations. Therefore, new controlled and reproducible models and protocols are highly warranted.

The ideal experimental stimulus to elicit gut pain in man should be natural, minimally invasive, reliable in test-retest experiments and quantifiable. Preferably the pain should mimic the observations in diseased organs by evoking phenomena such as allodynia and hyperalgesia[10,11]. The different methods for pain stimulation of the human GI tract are described below and in Table 1. Some of the existing stimulation methods seem to fulfill the above requirements but most laboratories use their own stimulation paradigms, often without the necessary standardization.

Table 1 The different methods for pain stimulation of the human GI tract

Stimulation modality	Stimulated structures	Advantages	Limitations
Electrical	Nerve fibers primarily in mucosa and muscle layers dependent of the stimulation intensity (not a specific activation of the nociceptors)	Excellent for repeated stimulation, suitable for neurophysiologic assessments of the pain	The electrical threshold depends on the fiber diameter, i.e. small-diameter nerves cannot be excited without exciting others. May induce arrhythmias in areas near the heart
Mechanical	Mechanoreceptors located in different layers	Imitates a bolus, reproducible stimulus	Problems with estimating the transmural pressure and change in circumference
Thermal	Thermal sensitive receptors preferentially in the luminal layers	Activation of unmyelinated afferents in the mucosa selectively	Temperature stimuli in the range that can be felt are normally only relevant for sensation in the upper GI tract.
Chemical	Chemo-sensitive receptors, primarily in the mucosa	Resembles clinical inflammation, chemical stimuli activate predominantly unmyelinated C-fibres	Require a relative long latency time to the onset of effects, and that they are often not reproducible when repeated

Electrical stimulation

Depolarization of the nerve afferents by electrical current has been widely used as an experimental stimulus in humans for many years-for review see[9]. The electrical stimuli have proved to be safe in all parts of the gastrointestinal system[12-15].Various electrical stimulator devices connected to electrodes applied to the mucosa of the gut can evoke electrical stimulation. Stimulator devices can deliver different stimulation patterns, e.g., different waveforms, frequencies, and duration of the stimulus. This activates with some selectivity different afferents and nervous structures, and hence evokes different kinds of pain[7]. The electrical stimulus intensity to evoke pain as well as the size of the referred pain area is reliable and reproducible[12,13,16,17]. The well defined onset and offset of the stimulation eliminates the latency to stimulation of the afferents seen with other methods[7] and makes it suitable to study pain mechanisms related to time[16,18]. Thus, the method is very suitable for neurophysiologic assessments of the pain[18-20]. Furthermore, the stimulation field is usually not as large as in other models, and diseases with localized pathology such as ulcers may better be mimicked[13,18]. Central integration or temporal summation, being a human correlate to the initial phase of "wind-up", is a potent mechanism for generation of referred visceral pain and can easily be evoked by electrical stimulation[16,18]. The major shortcomings are that electrical stimulation bypasses the receptors and activates the nerve fibres directly, and the method is not a specific activation of the nociceptors. The electrical threshold is related to the fiber diameter and one cannot usually excite small-diameter nerves without additionally exciting others. Furthermore, electrical stimuli may induce arrhythmias when areas near the heart are stimulated[18,21] and therefore bipolar stimulation where the electrical field is more localized may be recommended.

Previous methods have suffered from problems with insufficient contact between the electrodes and the gut, and in some studies pain was not evoked in all

subjects. We have improved the method by integrating the electrodes on the biopsy forceps for the endoscopes, which makes it possible to stimulate well defined areas in the esophagus, stomach, duodenum, terminal ileum and colon[13,14,16,18]. Recently we used a 6 mm nasal endoscope for the stimulations. Apart from a little unpleasantness associated with the intubations, all subjects were able to lie comfortably and none complained of unpleasantness due to the endoscope for up to 4 h. An example of stimulation of the esophagus is shown in Figure 1. The advantage of this modification is that the position of the electrodes is controllable and can be changed in case of stimulation of nearby somatic structures and nerves. The contact with the organ wall is secured and evoked motor phenomena such as secondary contractions can be studied. However, endoscopic stimulation demands special equipment and facilities and improvement of methods for "blind stimulation" is highly warranted.

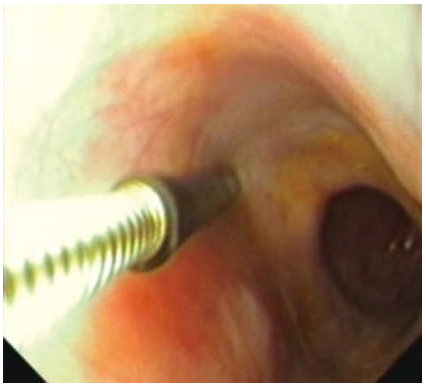


Figure 1 The stimulation electrode visualized in the distal esophagus using the nasal endoscope.

Mechanical stimulation

In the last decade several studies have addressed the mechanical and sensory function of the GI tract by means of mechanical distension. The mechanical properties of the GI tract are important for its function as a digestive organ and the gut contains mechanoreceptors at various locations in the wall, mainly in the muscle layers[6]. Mechanical GI tract stimulation, in particular balloon distension, has been widely used also to study GI smooth muscle tone[22-24], distensibility[22,25,26], peristaltic reflex responses[27], functional and organic disorders[28-32], referred pain and cerebral activation patterns[33] and the screening of new analgesics in healthy subjects and in patients with gut disorders[30,34-37]. There are several ways to stimulate the GI tract mechanically. Simple and physiological methods for distension of the gut such as ingestion of well-defined meals may be useful in clinical studies[38]. Balloon distension is, however, much more used than ingestion as mechanical stimulation method and easier to control. Therefore, the following sections will focus on controlled balloon distension methods used to evoke non-painful sensations and pain in the gut.

Most recent studies have used a method of measuring changes in volume of air in a balloon at constant pressure levels named "the Barostat". Several protocols and stimulation paradigms have been recommended for the Barostat, such as for example "phasic and tonic distensions". The stimulation paradigms have been thoroughly discussed recently and will not be described here-for review see[30,39]. The great advantage of the Barostat system and similar pressure-volume based methods are the relatively low cost, especially if the system is self-made. Furthermore, they are easy to use for routine purposes. Such systems have been used for determination of sensory and pain thresholds, and under different conditions attempts have been done to calculate the compliance and tension of the organs[26,30,39,40]. However, there are drawbacks with these simple methods. For example using the Barostat device, the data must be corrected for compressibility of air. The four major concerns, however, relate to (1) elongation of the balloon during distension; (2) the use of very long balloons; (3) the use of erroneous assumptions; and (4) the improper use of distension protocols. In more detail: (1) Balloon distension in a tube will cause the balloon to elongate to some degree due to the resistance to deformation in radial direction[41]. Volume is therefore not an accurate measure for the distension of the organ; (2) The use of long balloons is problematic because phasic contractions can be misinterpreted as muscle tone[42]. In addition relaxation in one end of the balloon and simultaneous contraction in the other end may not be registered as a change in volume at all; (3) Most previous studies have not validated the assumptions the data were based on. The most obvious and common mistake is to consider the fundus of the stomach to be spherical. From an anatomical and geometric point of view the stomach is not spherical and the wall structure with muscle layers in various directions indicates complex (anisotropic) properties. Distending a balloon in the fundus will deform the balloon into the corpus and antrum, resulting in a highly complex geometry; (4) No previous pressure-volume studies have considered the strain softening effect[43] and therefore it is highly questionable whether the results are reproducible. The tissue needs to be preconditioned before proper testing

The first three of the points mentioned above makes it impossible to compute the radius from the measured volumes. Consequently, estimation of wall tension using Laplace's law and computation of deformation in terms of strain measures will be invalid. It has also been a common mistake in gastrointestinal distension studies to consider the mechanoreceptors to be pressure receptors, volume receptors or tension receptors. Circumferential strain or stress are more likely candidates as the direct receptor stimulus, because the tensile stress and strain in distensible biological tubes are largest in the circumferential direction during distension[44]. Considering mechanical and receptor kinematic properties, strain is a more relevant parameter than stress (and tension). This is partly due to the fact that strain is a non-dimensional parameter that is independent of the original size of the organ and directly associated with tissue deformation. Recent studies clearly demonstrated that circumferential strain is an important determinant of mechanoreceptor mediated responses[45-48]. Correspondingly, studies providing tension calculations from Barostat studies have shown conflicting results, e.g.

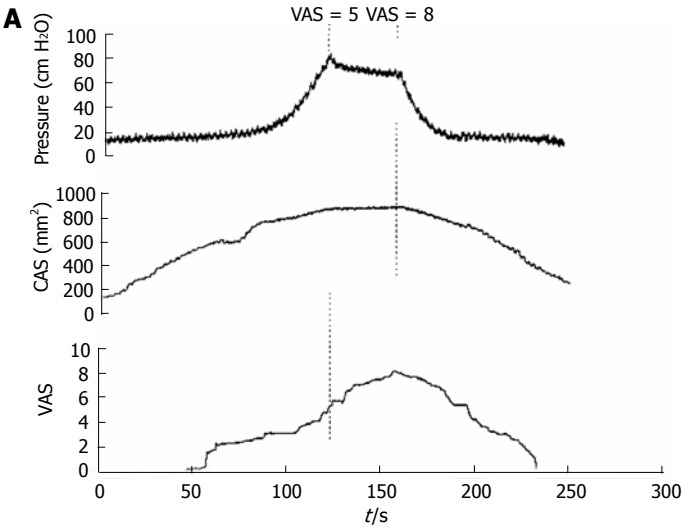


Figure 2 Top corner: The impedance planimetric probe for mechanical stimulation of the gastrointestinal tract. l and d denote infusion side for filling the balloon and the distance between two detection electrodes. Left: Recordings in the small intestine using the impedance planimetric probe with pressure, cross-sectional area (CSA) and VAS response as function of time during a ramp distension of the balloon up to the pain threshold where the bag volume is kept constant. It appears that the pressure decreased, the CSA was constant whereas the pain continued to increase.

in a recent study of the stomach the estimated tension seemed to correlate with the sensation[26,49], whereas another study[35] showed a highly inter-individual variability in the sensation score to the applied tension, suggesting that other factors than wall tension influence the sensation. However, uncertainties in the assumptions given above, and lack of proper geometric and biomechanical considerations can also explain these findings[41].

Newer methods based on impedance planimetry (Figure 2) allows recording of the luminal cross-sectional area directly and estimation of the radius in the distended segment of the esophagus or intestine[17,45,47,48,50,51]. From the radius it is possible to compute the circumferential wall tension and strain with more accuracy than from volume measurements[44]. Circularity must be assumed but this assumption has often been validated[44]. Finally, mechanical distension combined with ultrasound methods may offer the possibility for a better anatomical characterization and computation of stress in the GI tract[52]. For the stomach and other organs with complex geometry, imaging methods that provides data on the surface curvatures (3D-ultrasound, multislice CT-scanning or MR-scanning) in the whole organ may be used together with numerical mechanical analysis such as finite element analysis.

Thermal stimulation

In the skin rapid heating activates first myelinated Aδ-fiber, where the evoked sensation corresponds to the "first pain felt within 0.4 s after the heat stimulus. The first pain is followed by a C-fiber mediated second pain which is less well localized and of longer duration, being described as 'throbbing, burning or swelling'[53]. Slow heating gives a preferential activation of the unmyelinated C-fibers and the best evaluation of second pain[54]. Short lasting thermal stimuli have also been used in the human GI tract. Here they are believed to activate unmyelinated afferents in the mucosa selectively as opposed to mechanical and electrical stimuli, which activate afferents in both the superficial and deeper layers[6]. Thermosensitive mucosal afferents throughout the gut have been demonstrated in animal studies[6], and in the human esophagus, stomach and rectum[6,17,55-57]. The major limitation of these models is that temperature stimuli in the range that can be felt are normally only relevant for sensation in the upper GI tract. On the other hand, although temperature receptors may have less clinical relevance, characterization of the response to stimuli in the most superficial layers of the gut may be of major importance to develop a comprehensive sensory evaluation[4]. In recent studies the temperature of re-circulating water was continuously measured inside a balloon positioned in the esophagus[17,58]. The temperature stimuli showed a nearly linear stimulus-response relationship, demonstrating the validity of the receptor activation.

Chemical stimulation

Chemical stimulation of the GI tract more closely resembles clinical inflammation and is believed to approach the ideal experimental visceral pain stimulus[9]. Such stimuli have successfully been applied to the skin and muscles[11,59,60]. In contrast to electrical stimuli, chemical stimuli activate predominantly unmyelinated C-fibres[61] and this may be an advantage in basic studies of differentiated nerve populations. Acid stimulation of the esophagus is the most used method to sensitise the gut[59,62-65] but chemical stimulation of the gut with alcohol, bradykinin, glycerol, capsaicin and hypertonic saline were also done in humans[18,55,58,66-69]. For details see[4]. The major disadvantage of chemical stimuli is that they require a relative long latency time to the onset of effects, and that they are often not reproducible when repeated[9]. Thus, although chemical stimulation may evoke pain, the major relevance of the model is in sensitization of the visceral afferents to subsequent experimental stimulation.

Ischemic stimulation

Ischemia has been widely used for induction of muscle pain. Ischemia is important in many diseases such as mesenteric ischemia and ischemic colitis. Ischemia has been used in animal models of visceral pain[9]. Such studies are not acceptable in humans and unfortunately no human experimental gut models exist. However, distension of the gut may result in diminished blood flow. Accordingly, Öhman *et al*[70] observed a significant drop in blood supply during distension of balloons in the small intestine in rats.

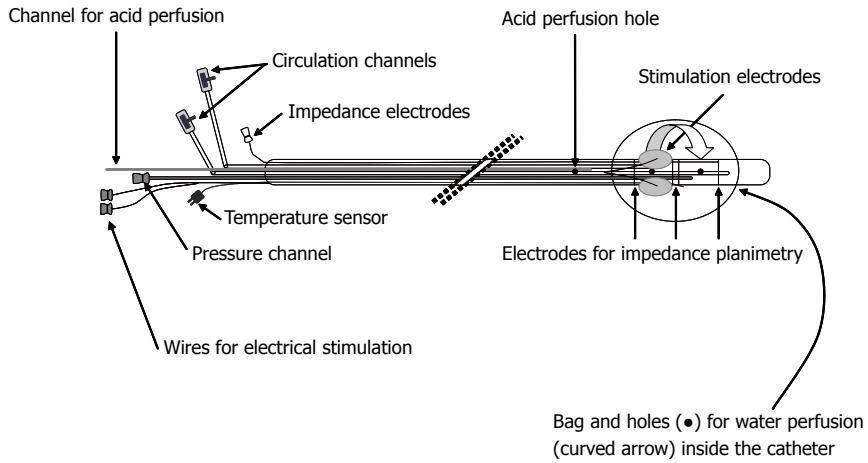


Figure 3 Schematic illustration of the probe used for multi-modal (electrical, mechanical, cold and warmth stimuli) of the esophagus. The stimulations can be combined with sensitization using acid perfusion of the distal esophagus.

Very recently, Hoff and coworkers presented a multimodal probe that selectively was developed for differentiation between mechanical and ischemic pain mechanism[71].

Multimodal stimulation of the GI tract

As pain is a multidimensional perception it is obvious that the reaction to a single stimulus of a given modality can represent only a limited fraction of the entire pain experience. The possibility for combining different methods for stimulation and assessment will approximate the clinical situation where many different nerves and pathways are activated. Thus, the method will give a more comprehensive and differentiated information about the nociceptive system compared to stimuli using single or a few modalities[72]. For comprehensive experimental studies mimicking the clinical situation, a multimodal testing approach must therefore be used, where the test battery will increase the probability for activation of a range of relevant nervous mechanisms. Such sophisticated methods will be able to select the best test procedures to explore different basic aspects of pain as well as pharmacological modulations. Multimodal models have shown their value in somatic pain models, where single modality models have been inadequate to test for example pathophysiological changes and effects of specific drugs[73,74]. Especially if the stimulation includes modalities known to evoke peripheral as well as central sensitization, the likelihood that a model will mimic clinical pain is high despite the non-harmful nature of the stimulation[73,75]. In somatic models it is possible to give many different stimuli and evoke central phenomena (e.g. central integration of summated stimuli) of clinical relevance (for review see[11,59]). In the GI tract, however, difficulties with access to the organs and technical limitations of the currently available models have until now made such a multimodal stimulation approach difficult. Some authors have combined mechanical and electrical stimuli[12,75,76] or mechanical and chemical stimuli[77,78], and in a recent study in the esophagus mechanical and electrical stimuli were combined with sensitization to acid[79]. We recently introduced a multimodal model with combined mechanical, electrical, cold and warmth stimuli of the esophagus together with acid sensitization[17,58] (Figure 3). The multimodal approach gives the possibility for a differentiated stimulation of receptors in the superficial and deep layers of the gut. The possibility for induction of hyperalgesia with e.g., acid perfusion and evoking central phenomena such as summation, allodynia and referred pain makes the model clinically relevant with respect to increase the knowledge of peripheral and central pain mechanisms[64,65,80]. The pain assessment should ideally also be multimodal and for example include quantitative and qualitative sensation, assessment of referred pain and neurophysiologic measurements. For details see[4]. However, is will typically not be feasible with too many stimulation and assessment parameters.

METHODS FOR AMPLIFICATION OF THE CENTRAL NEURONAL RESPONSE

Chronic pain is characterized by modifications of the central nervous system such as central sensitization[81]. Hence, decreased thresholds to normal stimuli such as stools and air in the GI tract seem to contribute to many of the symptoms reported by patients with inflammatory and functional diseases in the gut[82,83]. Phenomena relating to sensitization of the central nervous system (CNS) are enhanced responses to repeated stimulation, allodynia/hyperalgesia and increase in the referred pain area[4]. Repeated electrical or mechanical stimuli to the small and large intestine[16,84,85] may cause integration of the neuronal response and thus pain, and this may be used as a model for enhanced central gain (Figure 4). Munakata *et al* showed the importance of central mechanisms in functional gut disorders[86]. In their study the patients developed rectal hyperalgesia following repetitive sigmoid distensions. Induction of hyperalgesia in one area of the GI tract following conditioning of the more proximal parts most probably results from central sensitization. Chemical stimulation may also evoke sensitization. Acid stimulation of the esophagus has been used as a chemogenic stimulus by several authors[76-79,87]. Increased response to mechanical, electrical and thermal stimuli after the chemical sensitization have been demonstrated[58,76,78,79], although previous studies using latex balloons were not as consistent. This may be related to methodological problems using latex balloons, where the distension data need to be corrected for the balloons intrinsic mechanical properties *etc*.[44,78]. Sarkar *et al*[79] demonstrated the relevance

of central mechanisms in the gut, as acid exposed to the esophagus not only resulted in local allodynia, but also sensitized the proximal, non-acid exposed part of the organ to electrical stimuli. Capsaicin also increases the sensation in the esophagus to acid reflux[88,89] and when capsaicin was applied to the small intestine, consistent hyperalgesia to mechanical stimuli was seen[18]. Lipid infusion in the duodenum has also been shown to induce allodynia and increase the referred pain area to distension in functional disorders[90,91]. As fat is a natural stimulus to the gut, such studies may increase the understanding of the postprandial symptoms in these patients.

EXPERIMENTAL PAIN AND GASTRO-ESOPHAGEAL REFLUX DISEASE

Gastro-esophageal reflux disease (GERD) is defined as chronic mucosal damage or typical symptoms, which reduce quality of life by the abnormal reflux of gastric contents into the esophagus. GERD is very common in the population with up to 30% of the European population reporting heartburn and/or acid regurgitation during the previous 12 mo[92]. Recent studies revealed that up to 70% of the GERD patients have non-erosive reflux disease (NERD) according to endoscopy, 24-h pH profile and symptom-reflux association indices. However, in patients with NERD, the quality of life impairment is comparable to that in patients with erosive esophagitis[93]. The symptoms in reflux disease are highly variable and poorly understood. Thus, in patients with GERD no simple relation seems to exist between the symptoms and severity of the disease[94-96]. Although treatment with proton pump inhibitors (PPI) is very effective, many patients continue to have symptoms despite treatment[97,98], and in a recent study 50% of the patients continue to have pathologic reflux despite effective symptom control with PPI[99]. Furthermore, it is estimated that 30%-60% of patients with NERD will have normal ambulatory 24-h esophageal pH monitoring and thus no effect of PPI treatment[93]. Although some of these patients may have reflux of non-acid gastric contents, it is still not clear what causes the symptoms in many patients.

Experimental pain methods have contributed to the understanding of the symptoms in reflux disease. In an animal study Garrison *et al*[100] demonstrated that spinal neurons in the cat receiving input from the distal esophagus also received convergent input from the thoracic wall and the heart. When the esophagus was sensitized with turpentine, the neurons responded to a smaller mechanical stimulus from the different sites. Such data gives evidence that central mechanisms may explain the symptoms in a substantial proportion of the patients. In humans, however, relative few studies have been done to explore the pain mechanisms in reflux disease.

Non-erosive reflux disease

Peripheral pain mechanisms: Assessment of mechanosensitivity using intra-esophageal balloon distension has yielded contradictory results. In NERD patients Rodriguez-Stanley *et al*[101] reported a decrease in sensation and pain thresholds to distension compared with a historic control group. Trimble *et al*[102] studied NERD patients without excessive reflux and found that these patients were most sensitive to esophageal balloon distension, whereas patients with excessive reflux had a level of sensitivity similar to that of healthy control subjects. In another study using esophageal balloon distension delivered by an electronic barostat, patients with NERD and patients with erosive esophagitis did not demonstrate an increase in mechanosensitivity when compared to normal controls[87]. The previous experimental pain studies used mechanical stimulations based on recording of volume and pressure. As stated above these studies may lead to errors relating to the deformation field and erroneous conclusions[47]. Is has been suggested that chronic esophageal exposure to excess acid affects chemosensitive but not mechanosensitive afferent pathways[87] and that the key abnormality in NERD patients is that they are hypersensitive to acid reflux. Recently we conducted a study in patients with NERD where 50% had a positive 24-h pH measurement. Furthermore a multimodal approach was used. Our data showed that the patients had hyperalgesia to heat stimulation, whereas they were hyposensitive to mechanical stimulations. There was a difference in the NERD subgroups as patients with a pathological pH profile exhibited hyposensitivity to mechanical stimulations compared to both controls and patients with normal pH monitoring (Figure 5). Taken together with the above findings the results reflect that patients with pathological acid reflux may be less sensitive to mechanical stimulation and more sensitive to heat. The selective sensitization to heat may be related to specific receptor activation. Recently, we showed that acid perfusion of the esophagus in healthy subjects differentially sensitizes the esophagus to heat, but not cold stimuli[80]. The VRI receptor may be important for our findings. The receptor can be activated by a variety of stimuli, including acid (protons) and increases in temperature that reach the noxious range. Thus, it can be hypothesized that in patients with NERD the acid reflux and resulting peripheral sensitization

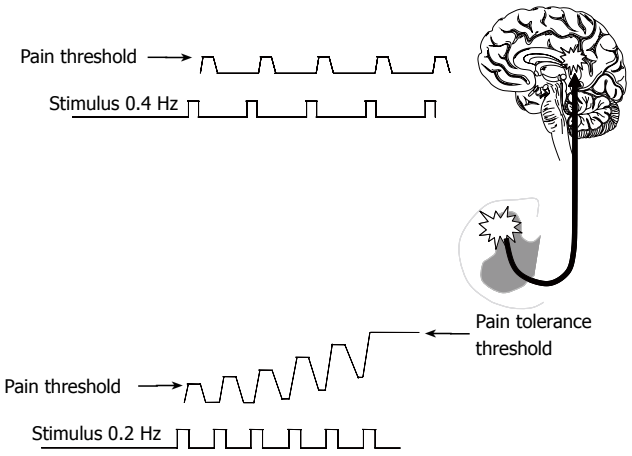


Figure 4 Central integration of repeated stimulation is seen when the stimulation frequency exceeds 0.3 Hz (bottom picture), whereas slower stimulation does not change the central gain (top picture).

results in a significant change in the sensation to heat stimuli working on the same receptor as the acid. Further studies need to explore whether the VRI receptors are up-regulated as seen in patients with erosive disease of the esophagus[103]-see below.

Central pain mechanisms: In the studies described above we also found an increase in the referred pain areas for both mechanical and heat stimulation in NERD patients. Previously we showed that acid perfusion of the distal esophagus in healthy subjects resulted in an increase in the referred pain area to differentiated esophageal stimuli[58]. This is most likely related to central neuronal hyperexcitability after acid perfusion, and subsequent opening of latent connections between converging neurons from visceral and somatic structures in the CNS[81]. Thus, the larger and widespread localization of the referred pain area is thought to represent central hyperexcitability. Consistent with these findings Penagini *et al*[104] showed that patients with NERD had increased sensitivity to distension of the proximal stomach. This viscero-visceral hyperalgesia is also considered a central phenomenon[105]. Experimental acid perfusion of the esophagus in healthy subjects has also been shown to increase the amplitude of the polysynaptic nociceptive reflex working at the spinal level[58]. Sarkar *et al*[79,106] demonstrated that acid perfusion of the distal esophagus resulted in allodynia and shorter latencies of the evoked brain potentials to electrical stimulation of a more proximal segment of the esophagus. Accordingly, we recently found a backward shift of the early activity in the cingulate gyrus to esophageal pain stimuli after acid perfusion of the organ[107]. Thus, there is substantial evidence that exposure of acid in the esophagus such as in some NERD patients may result in central neuroplastic changes at spinal and supraspinal levels.

Erosive GERD

Peripheral pain mechanisms: Patients with erosive disease of the esophagus may have a more severe disease than NERD patients, although erosive disease may also be a distinct entity[108]. In a study comparing patients with esophagitis with controls, Fass *et al*[87] demonstrated enhanced perception to acid perfusion, but the response to mechanical stimulation was normal. Such findings may point towards a differential effect on mechano-sensitive and chemo-sensitive pathways in esophagitis. Recently, we compared the sensory response in patients with grade B esophagitis and healthy controls using the multimodal approach described above. The patients had hyposensitivity to the mechanical stimulations, but had hyperalgesia to heat. These findings were comparable to those in NERD patients with abnormal pH profile and indicate that the pain mechanisms may be the same whenever erosions occur or not. A recent paper suggested that abnormal tissue resistance to acid may explain both the hyperalgesia and motor abnormalities seen in many patients with GERD and NERD[109]. In our studies the patients showed hyperalgesia to heat (but not cold) stimuli[110]. We believe that VR1 receptors sensitized by the acid reflux are important, and VR1 receptors have recently been shown to be up-regulated in esophagitis[103].

Central pain mechanisms: Central changes are also believed to be important in erosive disease. Although Fass *et al*[87] found a normal location of the referred pain we recently showed that the size of the referred pain area was larger than in controls. Thus, there is substantial evidence that exposition of acid in the esophagus in patients with esophagitis results in central neuroplastic changes. One can speculate that the reason for specific hyposensitivity to mechanical stimulations in patients with erosive disease may be related to well functioning counter-regulatory neural mechanisms acting from the brain stem at the spinal cord level. These may prevent the development of long-lasting sensitization of mechanosenstive afferent pathways[111]. The central pain modulating systems rely on a balance between facilitatory and inhibitory descending pathways and intrinsic spinal circuits and is not predictable in the individual patient[112,113]. Studies on animals have shown that the system is an important mechanism in the modulation of visceral stimuli[114] and these neuroplastic changes may result in increased referred pain on the one hand and dampening of the activity from mechanosensitive pathways on the other. In general chronic tissue injury and pain has been associated with higher thresholds to mechanical stimulation in different regions of the gastrointestinal tract. For example, chronic inflammation of the small bowel[115] in patients with inflammatory bowel disease is not associated with mechanical hyperalgesia of the rectum. This is contrasted by the pain in functional visceral disorders where hyperalgesia and allodynia to mechanical stimuli of the gut are typically found[86,115-118]. Hence, it can be speculated that a difference in the balance between noxious control systems arising in the brainstem may explain the findings in the different patient groups.

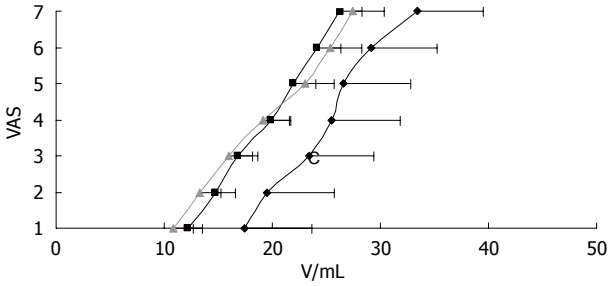


Figure 5 Stimulus-response function (mean ± SEM) to mechanical stimulation in patients with non-erosive gastro-esophageal reflux disease (NERD). The controls (■, *n* = 15) and patients with a normal 24-h pH-profile (▲, *n* = 7) had similar response to the mechanical stimulation, whereas patients with pathological pH-profile (◊, *n* = 6) showed hypoalgesia to the stimulations. The x-axis shows the volume of the balloon in the distal esophagus and the y-axis denotes the pain response on a visual analogue scale (VAS) with 5 as the pain threshold.

Non-cardiac chest pain

Non-cardiac chest pain (NCCP) was a term invented by the cardiologists. It was defined as "angina-like" chest pain without demonstrable abnormalities in the coronary vasculature. The more broad term "unexplained esophageal chest pain" is probably a better definition in gastroenterology, and may be defined as chronic chest pain, which is most likely of esophageal origin, but where

conventional tests for cardiac, esophageal, pulmonary (pain can be evoked from the pleura only) and musculoskeletal disorders are negative. The pain can be angina-like, but can also mimic other chest pain disorders such as gastro-esophageal reflux disease and musculoskeletal disorders. However, in the following the term NCCP will be used as it is the most common description of the syndrome. NCCP is very common especially in the younger age groups and the prevalence ranged from 22%-30% in the general population[119,120]. However, many persons have only modest and intermittent complaints and such cases must be classified as intermittent chest pain that is of no major burden, and a rough estimate would be that severe symptoms are seen in 2%-3% of the population.

If the patients describe "heartburn" and "acid regurgitation" they are typically classified as having GERD. However, burning pain may also be evoked by e.g., experimentally distension or electrical stimulation of the stomach[121] and the value of the subjective description of chest pain is up to discussion. GERD is also the most frequent cause of NCCP[122,123]. In this case the chest pain is no longer unexplained and the overlap invalids the diagnostic criteria for NCCP. The reason for heartburn in GERD is also up to discussion. Fass *et al*, have demonstrated that acid is not the only visceral stimulus that leads to heartburn[87], and recently Balaban *et al*, suggested that longitudinal muscle contractions of the esophagus as detected by high frequency intraluminal ultrasound, may be the motor equivalent of heartburn sensation[124]. Thus there is major overlap between the symptoms relating to the different chest structures.

The origin of chest pain

The symptom description is consistent with the organization of the nerves supplying the esophagus and the chest. The visceral afferents converge on a large scale with neurons that receive input from superficial and deep somatic tissue as well as other viscera[105]. Accordingly, in the thoracic spinal cord more that 75% of all neurons receive both somatic and visceral information, being contrasted by the actual number of visceral afferents (5%-15% of the inflow) entering the dorsal roots[6,125]. The visceral afferent terminals are widely distributed in the spinal cord and some organs have a wide spinal representation spreading to several segmental levels. For example the esophagus is innervated craniocaudally from the mid-cervical to the second lumbar segments[6]. Therefore a few visceral afferent fibers can activate many central neurons. Through the extensive functional convergence of somatic and visceral nerves on the same central neurons this wide activation of the CNS may explain the diffuse and unpleasant nature of visceral pain together with referred pain to somatic structures and other viscera[126]. In the clinic the above considerations typically leads to a very diffuse and heterogeneous description of the symptoms and experimental pain models may shed light over the pathogenesis of NCCP and related symptoms.

Peripheral pain mechanisms

Experimental pain studies in patients as outlined in the Introduction section have the possibility to control the stimulus and assessment parameters, and thus explore the abnormal pain system in further detail. Most studies found that the sensation and pain detection thresholds to distension, electrical and acid stimuli of the esophagus were lower in NCCP patients compared with thresholds found in healthy subjects[76,79,127-132]. However, the reliability of the test for diagnosing NCCP has been questioned[4], and in some experiments only few patients had abnormal sensation[133]. There are, however, severe methodological problems relating to these studies. Early balloon distension studies were based on simple volume and pressure measurements using latex balloons. As stated in the section above "Mechanical stimulations" this caused large errors due to the deformability of latex and lack of control of the stimulation field. There are also limitations and sources of error with the systems based on volume and pressure. The use of methods based on impedance planimetry has encompassed many of these limitations. Rao *et al*[134] used impedance planimetry and showed that patients with NCCP suffered from esophageal hyperalgesia. Furthermore the esophagus was more "irritable" than in controls, as the distensions resulted in more vigorous contractions at lower pressures. In more recent studies hyperalgesia was also found after relaxation of the smooth muscle with atropin[135], and there was evidence that the lower segments of the esophagus exhibited a higher level of sensory dysfunction compared to higher segments[136]. These studies were based on phasic distensions, which did not take the strain softening effect into account. Hence, we recommend to preconditioning the tissue by several distensions until the stress-strain relationship becomes reproducible[17,45,47].

Central pain mechanisms

Paterson *et al*[137] showed that repeated distensions conditioned the esophagus in NCCP resulting in higher pain scores. The effect of repeating stimulations has also been demonstrated in patients with irritable bowel syndrome, and probably this is a phenomenon relating to central hyperexcitability[86]. In a recent study we used a ramp distension protocol to distend the distal esophagus in patients with NCCP before and after sensitization with acid[138]. Patients with NCCP did not seem to be more vulnerable to develop esophageal hyperalgesia to the slowly increasing mechanical distensions as compared to controls. However, there was evidence for abnormal central pain processing as there was an increased and widespread referred pain area to the mechanical stimulations (Figure 6) and the patients were sensitive to repeated mechanical stimulation. Furthermore, after acid perfusion (believed to evoke hyperexcitability of central pathways mainly) there was a major sensitization to the distensions. Thus, it was concluded that NCCP patients showed facilitated central pain mechanisms, which may explain the character of their symptoms. This may have important implications for the diagnosis and treatment in these patients, where drugs with central effects should be used in the treatment.

Several studies have focused on brain activation to experimental pain stimuli in NCCP. The

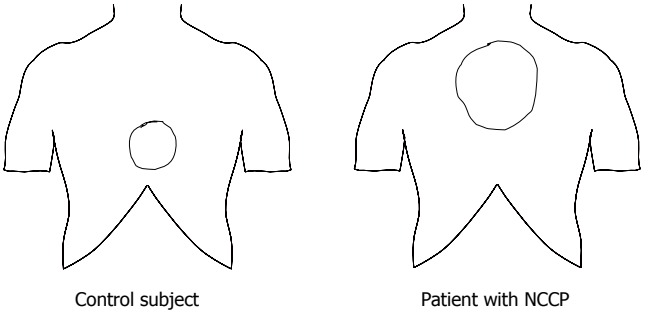


Figure 6 The localization and size of the referred pain area to mechanical stimulations of the distal esophagus in a typical control subject and a patient with non-cardiac chest pain (NCCP). The patient had and increased size and abnormal localization of the referred pain.

electroencephalography monitors the brain activity to external stimuli directly in real time. When a repetitive stimulus is applied and the cortical electrical activity is averaged (time-locked to the stimulus), the stimulus evoked cortical potential (EP) can be extracted from the background electrical activity and is shown in shape of a waveform with different peaks. Each peak in the EP represents a synaptic event associated with the transmission of afferent information from one group of neurons to another. The early peaks are supposed only to be influenced by the stimulation rate, intensity and localization, and they reflect to a major degree the brain loci that process the pain intensity and localization[139,140]. Three studies showed lower EP amplitudes to esophageal electrical and distension stimuli in patients with NCCP compared with healthy subjects[127-129]. Furthermore, Hollerbach *et al*[128] found shorter latencies of the EP peaks to electrical esophageal stimulation and more pronounced changes of the outflow of the autonomic nervous system in NCCP patients. Frobert *et al*[141] stimulated additionally the sternal skin (referred pain area) and showed shorter latencies and lower amplitudes of the EP to skin stimulation in NCCP patients. These findings suggest an increased central nervous system response to visceral stimuli in patients with NCCP comparable to those found in other groups with functional bowel disorders, e.g. patients with irritable bowel syndrome. Recently, Hobson and Aziz [142] suggested that different subgroups of patients with NCCP may exist: (1) Those with short latency of the early EP components having sensitization of gastrointestinal afferent pathways, and (2) those with long latencies and enhanced late responses reflecting hypervigilance and increased affective processing.

CONCLUSION

Pain is the most prevalent symptom in gastroenterology but yet poorly understood. This is reflected in the treatment of visceral pain that is often very difficult and highly challenging. Methods to evoke and assess experimental pain in the GI tract have recently been improved and used to explain the pain mechanisms in health and disease. In diseases relating to the esophagus these methods have contributed to our understanding of the symptoms reported in these patients. Especially does abnormal activation and plastic changes of central pain pathways seem to play a major role. These findings may lead to an alternative approach for treatment in patients that does not respond to conventional medical or surgical therapy, and probably analgesics with effect on the central nervous system such as tricyclic antidepressives, serotonin reuptake inhibitors, ion-channel blockers or opioids with additional effect on the N-methyle-D-aspartate (NMDA) receptor or kappa- opioids receptors should be used. Phase II trials where the experimental methods are used to evaluate these drugs will be a feasible way to obtain more knowledge, before more expensive large scale phase III studies in the clinic are initiated.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, Asbjorn Mohr Drewes, MD, *Series Editor*

Functional oesophago-gastric junction imaging

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Abstract

Despite its role in disease there is still no definitive method to assess oesophago-gastric junction competence (OGJ). Traditionally the OGJ has been assessed using manometry with lower oesophageal sphincter pressure as the indicator. More recently this has been shown not to be a very reliable marker of sphincter function and competence against reflux. Disorders such as gastro-oesophageal reflux disease and to a lesser extend achalasia still effects a significant number of patients. This review looks at using a new technique known as impedance planimetry to profile the geometry and pressure in the OGJ during distension of a bag. The data gathered can be reconstructed into a dynamic representation of OGJ action. This has been shown to provide a useful representation of the OGJ and to show changes to the competence of the OGJ in terms of compliance and distensibility as a result of endoluminal therapy.

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Key words: Oesophagogastric junction; Lower oesophageal sphincter; Competence; Distensibility; Functional imaging

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INTRODUCTION

The oesophago-gastric junction (OGJ) is located between the oesophagus and the stomach. Its dynamic action is dependant upon the intrinsic and extrinsic effects of its own anatomical structure and its position with respect to the surrounding organs in this region of the body. Despite its relationship to diseased states of the oesophagus, no objective measurement technique has been developed to quantify the normal or abnormal functioning of the OGJ. It is generally accepted that sphincters divide the gut into functional segments. They are characterized by a resting tension, generally known as muscle tone which is greater than in the two adjacent segments. It is common nowadays to give the term sphincter a functional meaning rather than its original anatomical meaning[1]. The sphincter at the lower end of the oesophagus, the lower oesophageal sphincter (LOS), forms part of the OGJ structure. At the OGJ the oesophagus opens into the stomach at the cardia. It is important that backflow of stomach secretions into the oesophagus is controlled at the OGJ. The OGJ guards against gastric acid moving up into the oesophagus, opening only transiently to allow passage of the swallowed food into the stomach. Swallowing is a demanding reflex activity[2]. The diaphragmatic hiatus, through which the oesophagus passes just at the OGJ, has a role in this valvular mechanism[3].

Several other structures are important in maintaining a barrier in the OGJ against the back flow of food and the stomach acids into the oesophagus, known as the anti-reflux barrier. The intrinsic muscles of the distal oesophagus and the sling fibres of the proximal stomach make up the internal mechanism of the LOS[4]. The muscles of the diaphragm that connect to the OGJ make up the crural diaphragm and this constitutes the external LOS mechanism. The tissue that connects the distal oesophagus to the crural diaphragm is known as the phreno-oesophageal ligament[5].

The OGJ can be distinguished from the body of the oesophagus by its behaviour pattern characteristics. There is an increase in the tone of the circular muscle in this region. The sphincter relaxes in response to a swallow and this usually precedes the arrival of a contraction wave travelling down the oesophagus. This phase of relaxation is followed by a short-lasting elevation of pressure above resting values[6]. In recent years it has been identified that the sphincter sometimes relaxes even when a swallow does not occur. These relaxations are know as transient lower oesophageal sphincter relaxations (tLOSRs)[7]. Other factors contribute to the role of the OGJ in controlling the flow of solids, liquids and gases between the oesophagus and the stomach. Pressures in the abdominal and thoracic cavities are involved as well as the exact intra-abdominal

location of the junction. Time and posture are also important factors.

THE ROLE OF THE OGJ IN DISEASE

Disorders of the OGJ in general relate to two diseased states. Conditions where the disease may be related to an OGJ not closing normally are generally referred to as gastro-oesophageal reflux disease (GORD) and conditions where the OGJ fails to relax or open, which is referred to as achalasia.

Gastro-oesophageal reflux disease

GORD symptoms are very common in the general population affecting up to 44% of the US population at least once a month and 20% of the population once a week[8,9]. It has been shown that patients suffering from GORD have a lower health related quality of life than patients with angina and mild heart failure[10]. There is solid evidence that incompetence or dysfunction of the OGJ is a primary determinant of GORD. Three mechanisms at the OGJ lead to an increased number of acid reflux events associated with GORD: tLOSRs, free reflux during periods of low LOS pressure or deglutitive relaxation of the LOS[7,11-13]. Much more needs to be learned about the dynamic action of the OGJ if we are to fully understand its role in GORD. Current treatment of GORD is with acid suppressing drugs such as proton pump inhibitors but for those refractory to pharmacological treatment surgery is often recommended. The surgery of choice for GORD is the laparoscopic Nissen fundoplication[14].

Achalasia

Achalasia is an oesophageal disorder of unknown cause characterized by aperistalsis of the oesophageal body and impaired relaxation of the lower oesophageal sphincter[2,15]. It is a relatively rare disease and in Western countries the annual incidence is in the range 0.5 to 8 cases per 100 000 people[16]. It can be difficult to make a diagnosis in some patients because of vague symptoms. Symptoms can emerge slowly and patients adapt. The diagnosis can be confirmed by endoscopy and manometry[17]. Developing a device that can profile the OGJ will contribute greatly to the understanding of this disorder, which often manifests as a permanently constricted or toned sphincter. Surgical performance of an oesophageal myotomy is the treatment of choice for some severe cases of achalasia[18]. Currently the laparoscopic oesophageal Heller-Nissen procedure is commonly used[19].

BIOMECHANICS OF THE OGJ

Biomechanics are the laws of mechanics applied to biology[20]. There is an increasing recognition of the importance of tissue biomechanical properties for function, e.g. organ geometry, resting conditions and elasticity is determinants of flow in the organ and sensory responses in the neural circuits. When dealing with biological tissues, solid mechanical parameters such as the relation between forces (membrane stress resultant, tension) and deformation (often expressed as a strain) are important to evaluate. The mechanical properties of the OGJ are important determinants of its distensibility and competence. In vitro studies have shown potential in measuring biomechanical parameters in the OGJ[21-25]. Very few methods exist for assessment of mechanical properties in the GI tract *in vivo*. One method is impedance planimetry from where the cross-sectional area (CSA) and pressure data can be gathered and the wall tension and strain in the organ can be estimated[26]. Other methods rely on the combination of bag distension and manometry with high resolution imaging techniques such as 3D-ultrasound of the gastric antrum[27] or MRI of the rectum[28]. Manometry is of course a simple biomechanical measurement. Its use will be mentioned again later.

METHODS OF EVALUATING OGJ FUNCTION

In 1964 Mann *et al*[6] suggested that the zone separating the lower end of the oesophagus from the stomach can be recognized in two different ways, by its distinguishing anatomic features and its unique patterns of behaviour. A huge amount of knowledge about the anatomy of the OGJ has been assembled over the last 40 years, but much of our limited understanding of the behaviour has been related to manometric studies using single or multiple pressure recordings or a sleeve placed in the OGJ. While manometry provides useful information on motility within the lumens of the digestive tract, it may be more restrictive in what it can tell us about sphincteric regions such as the OGJ and their dynamics. Harris and Pope found that sphincters do not necessarily need to squeeze or contract tightly to be competent and therefore resistance to distension by measurement of radial force should be the prime determinant of sphincteric strength[29]. Measurement of luminal pressure, however, is not a direct measure of muscle tone and tension. It is merely a proxy of changes in force generated by the surrounding tissues[27,30].

No clinically acceptable quantitative method exists to directly measure OGJ mechanical function. Intraluminal pressure recordings is the only objective measurement technique for determining the competence of the LOS, but it is usually the more indirect method of measuring acid reflux in the oesophagus which is the key determinant of failure of the OGJ to inhibit flow. Thus, pH-metry rather than manometry is the recommended test for GORD[31].

Usually in medicine dynamic diagnostic imaging methods can be used to visualize and evaluate anatomical structures similar to the OGJ. Currently none of these methods give sufficient dynamic detail to contribute to the routine diagnosis and treatment of diseases of the OGJ. Recent developments in material sciences and technology have allowed the OGJ's resistance to distension to be studied using parameters such as distensibility, opening patterns and dimensions and flow rate[32-34]. It is becoming apparent that these measurements may be useful in determining the success of therapeutic interventions such as surgery[35]. However what is needed is a technique with high spatial and temporal resolution for determination of sphincter geometry and mechanical factors.

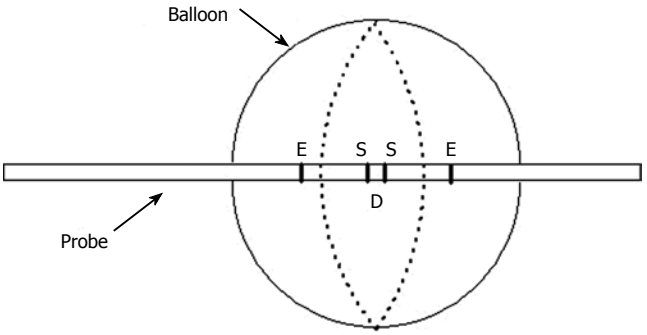


Figure 1 Sketch of a conventional impedance planimetry probe with a single set of excitation (E) and sensing (S) electrodes for the measurement of cross-sectional area in the middle of the bag. D is the distance between the sensing electrodes.

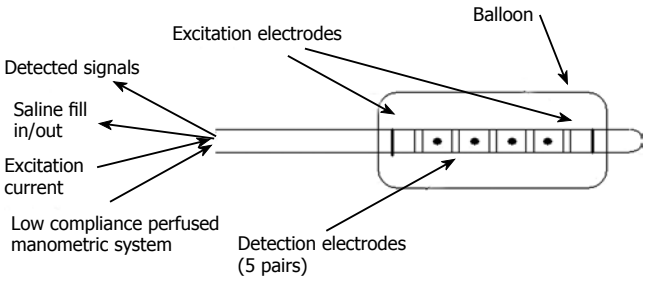


Figure 2 Diagram showing multi-electrode probe by Andersen et al for measuring 5 CSAs in the porcine rectum.

Impedance planimetry

Principle of Impedance Planimetry: Impedance planimetry (IP) is a technique established by Gregersen and coworkers for performing bag distensions in the GI tract (Figure 1). It has been used extensively to provide a single measure of CSA together with bag pressure in animal and human studies using electrical measurements inside the liquid filled bag[36]. Hence the term impedance planimetry. If we fill a polyurethane bag with saline and pass a constant current through it, the CSA is inversely proportional to the voltage across the sensing electrodes. If all other parameters are kept constant then the impedance becomes related to the CSA of the bag between the sensing electrodes.

$$V\alpha \frac{1}{CSA},$$

Many studies have shown the usefulness and reliability of IP in the oesophagus of healthy volunteers and patients[37-54]. Rather than analyzing distension data based on pressure-volume relationships as is the case with the "Barostat", IP enables data on the CSA of the bag during distension to be gathered. CSA shows a better relationship to the diametrical changes in the alimentary lumen than volume[27]. However, by far most studies have been done using a single CSA recording inside the bag rather than obtaining a series of CSAs. Due to previous limitations, it has only been used in a few studies in sphincter regions[55,56]. These limitations related to the difficulties placing the measurement electrodes at the point of most interest and the fact that distending a bag in a high-pressure zone tends to displace the bag during the measurements.

Multiple measurements: A recent pilot study in the porcine rectum demonstrates how an IP probe placed in the rectum with 5 sensing electrode pairs with 2mm between pairs and 2cms between sets can detect contractile events at different levels along the longitudinal axis of the gut (Figure 2)[39,57]. This multi-electrode technique demonstrated how a balloon with a conducting liquid and a constant current source set up across two excitation electrodes can measure multiple CSAs by measuring the voltage across multiple electrode pairs within the electric field.

There are a number of potential sources of error in multiple measurement IP systems. Electrodes placed too close together will interfere with each other. The number of wires required to make the electrical connections goes up and therefore the potential for interference, liquid leaking into the probe and bad electrical connections is much.

Manometry

Manometry has been used for more than 50 years to monitor motility in the gastrointestinal tract. Since 1977 reliable methods have been available to obtain accurate measurements of force of closure of the lumens of the intestine when contracting. These system often use reliable perfused hydraulic-capillary systems where pressure at a sensor outside the body can be used to accurately measure pressure at a point along a catheter via a low flow rate perfused channel[58]. This system has been used extensively to measure force via a pressure measurement in lumens and in liquid filled balloons. Errors in manometric readings can be caused by air bubbles in the capillary lines, occlusion of the measurement site and the compliance of the recording system but in general if well set up they produce accurate readings. In more recent years catheters with solid state transducers are becoming more popular for pressure reading and these overcome some of the measurement problems.

Using manometry to determine the competence of sphincteric or valvular regions in the lumens of the digestive tract is however dubious. Dent recognized the problem of positioning the measurement point 30 years ago and proposed a solution[59] and others tried to follow suit[60]. But pressure is a measurement of force over area and this may not be a proper measure of the function of a valvular region. As suggested by Pope and Harris we cannot assume that just because a sphincter is closed that it has a contract force or in the terminology of manometry that if it has a high lower oesophageal pressure it is deemed to be competent.

Calculation of wall tension

Law of laplace: For a membraneous cylinder with transmural pressure P and radius r the circumferential tension T can be given by :

$$Pr = T$$

Use of Laplace's law is based on the assumption that the geometry of the segment is circular and cylindrical and that the wall thickness is very small.

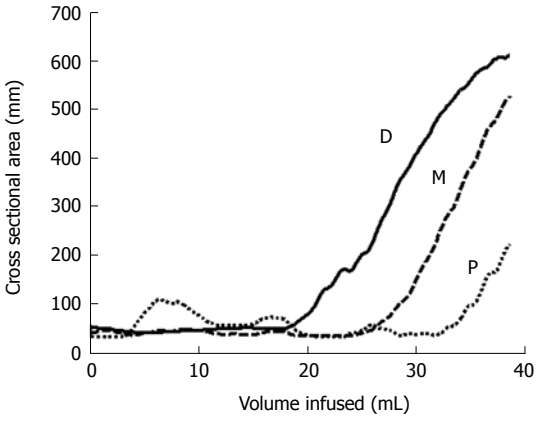


Figure 3 Plot showing the changes in the CSAs at the proximal (P), middle (M) and distal (D) electrode pairs as the balloon is distended at a constant flow rate indicated by the increase in volume.

Measurements using multi-electrode Impedance planimetry in the oesophagogastric junction

We have demonstrated that a multi-electrode probe with a cylindrical bag mounted and filled with saline can be placed in the OGJ and that data on a number of CSAs in this area can be measured[49]. Figure 3 shows results of 3 CSA measurements 8 mm apart measured from distension of a bag in the OGJ of a healthy volunteer. The plot shows how the most distal CSA (D) measurement increases more rapidly than the middle CSA (M) as a constant volume of saline is filled in to the bag. For the most proximal CSA (P) the converse is true. This suggests that distally the OGJ is more compliant than proximally. This work also demonstrated for the first time how wall tension in the OGJ can be estimated from simple distension measurements. This preliminary version of the multi-electrode technique demonstrated that a probe can be placed in the OGJ and that data can be successfully collected. However, there were limitations such as the fact that there were only three measurements made. A narrowing region such a sphincter can not be profiled very well with three CSA measurements unless the exact position of the sphincter is located and maintained. Another limitation is the outer diameter of the catheter on which the bag is mounted. In this case it was 4.5 mm. If the sphincter is to be distended from as close to the closed position as possible then a smaller catheter needs to be used.

Figure 4 shows how a probe capable of measuring 8 CSAs could be used to profile a sphincter region. It is quite obvious that a larger number of sensing pairs could be more useful in providing a geometric profile. Consequently a more elaborate probe for measurement of up to 8 CSAs in the region of the oesophago-gastric junction was developed. This probe can measure CSAs at 4 mm intervals through the OGJ thus spanning a measurement range of 28 mm.

Figure 5 shows the data from the 8 CSAs during a bag distension at a fixed flow rate of 40 mL/min to a volume of 60 mL. The information in this format can be difficult to decipher. A probe to make these measurements has been validated and test in a healthy control subject[57]. Since the OGJ is such a dynamic organ it is its functional activity which can be more of interest. Hence it is possible to generate an illustration of the geometric changes in the OGJ.

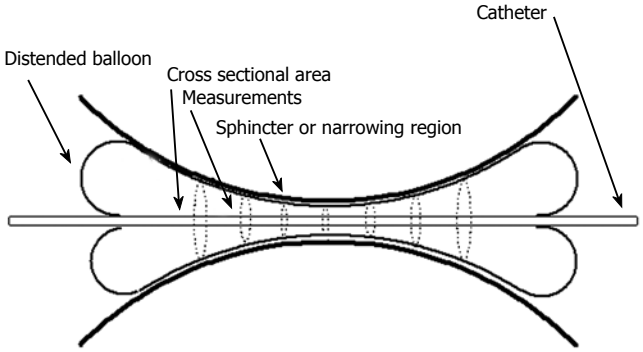


Figure 4 An example of how a probe which can measure 8 CSAs could profile the geometry of the narrowing region of a bodily sphincter.

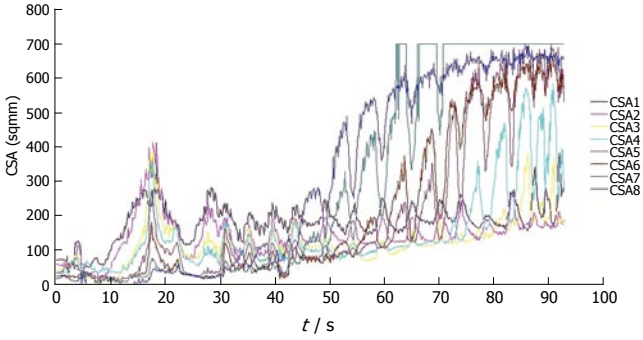


Figure 5 Plot showing changes in the 8 measured CSAs during bag distension at 40 mL/min. CSA1 is the most distal and CSA8 is the most proximal.

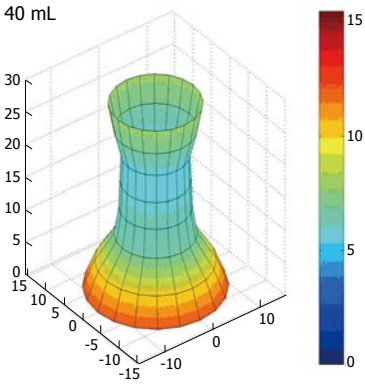


Figure 6 Three dimensional reconstruction of the OGJ geometry in a human volunteer after distension in to a volume of 40 mL. All dimensions are in millimeters. Colour change is indicating increased radius.

If we introduce the idea of demonstrating the CSA changes dynamically as reconstructed data we introduce a new concept known as the functional lumen imaging probe (FLIP). The concept suggests that by gathering data on the dynamic changes in the geometry of the OGJ during normal physiological changes we can represent these changes by recreating the geometry in a three-dimensional image. The FLIP technique can be used to monitor geometric changes in the behaviour of the OGJ during bag distension as can be seen in Figure 6. Here the dynamic data is reconstructed demonstrating the effect of the balloon distension on the amount of OGJ opening.

FLIP can quantify changes in compliance of the OGJ after endoscopic suturing at the OGJ in a pig model[61]. Figure 7 indicates how the geometric reconstruction can be used to indicate changes in the profile of the OGJ after endoscopic plication. The pressure is higher, the CSA is smaller and subsequently the wall tension has increased after the plications are inserted. These measurements indicate that the OGJ becomes less compliant after plication. No direct measure of the effects of these therapies on the OGJ has been demonstrated before. Recent studies have expressed concerns about the efficacy and durability of these procedures[62-65]. FLIP may in fact reveal new information on the mechanism of action behind these new reflux therapies. In effect it suggests that endoscopic suturing augments the compliance of the OGJ to make it less compliant. Perhaps patients could now be selected better for these procedures if their compliance can be confirmed to be abnormal in the first place and maybe the success of the procedure could be determined directly at the time of the procedure so for example more stitches can be added if necessary.

Since GORD patients are such a large group perhaps this is the most important area for FLIP to be developed. The probe can distinguish between patients for reflux surgery and normal controls. These are of course the extreme group of patients. Further studies are of course necessary on larger cohorts of patients and controls.

We have recorded with the FLIP the compliance of the OGJ intra-operatively during oesophageal myotomy for achalasia (as yet unpublished findings by the authors). FLIP offers a more sensitive and possibly a better tool than manometry and may be used for evaluating effects of surgery during the procedure as opposed to using manometry more as a blunt instrument before and after as used by Arain *et al*[66]. FLIP could provide a valuable new tool for the oesophageal surgeon.

Summary

Work already carried out shows for the first time that CSA and pressure measurements in the OGJ can be used to measure distensibility by an entirely new technique. It demonstrates how the already established technique of impedance planimetry can be used to measure geometry in vivo in the human oesophagus. It also shows that there is validity of these new measurements, their appropriateness for use in sphincteric regions in the body and their comparable accuracy has been shown with ultrasound. It has been indicated how the measurement could be used practically. Safety and efficacy has been demonstrated. The work also demonstrates how this new measure could be used as a functional imaging technique. Preliminary studies have shown potential applications of the probe. It could prove to be a very valuable tool in endoscopic therapies. It can distinguish between normal and a stitched OGJs in live pigs.

As a resistance to distension test this better describes the sphincteric action of the OGJ than tonic contraction. Even advanced manometry uses such as volume vector analysis, the Dent Sleeve or the Sphinctometer all rely on the principal that there is a force of contraction always present when the sphincter is closed[59,60,67-70]. With FLIP this assumption is not made and it presents more of a challenge test to the sphincter. The technique also represents a more direct technique to evaluate the OGJ using opening patterns with manometry and intraluminal impedance[71].

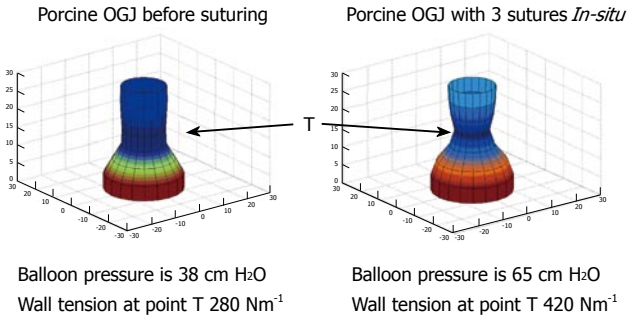


Figure 7 Geometric profile of the porcine OGJ before and after the placement of 3 sutures in the OGJ. T indicates the point where wall tension was measured.

Some other techniques for measurement of resistance to distension by other investigators are promising and confirm that the principals describe here have great potential for merit. However these other approaches are limited in a number of ways. The technique described by Pandolfino, although it is similar in its measurement design to FLIP, it requires fluoroscopic imaging[32,33,35]. It also depends on the pressure being controlled. Pressure control is difficult to achieve by increasing and reducing volume through a narrow catheter. Using pressure-volume data from the barostat to quantify distensibility does not a good basis in the application of the physical sciences. The bag on a barostat can expand in all directions we cannot be sure that an increase in the volume is reflected by a change in the geometry of the OGJ. This suggest that using this technique is of limited use[34]. Our findings in patients with achalasia confirmed this.

Perspectives

FLIP represents the first validated data on a probe capable of measuring OGJ compliance in a simple and time-saving procedure. It represents the opportunity to define the exact role of the OGJ in dysmotility and disease. As a new technique rather than representing a completed work it opens up a new door and perspectives on the junction whose function has been the subject of debate for many years. Future work and improvements in electronics and material sciences will mean that the resolution of the FLIP can be increased dramatically. Design materials have been carefully chosen to minimize signal error. The lower oesophageal sphincter or the oesophagogastric junction are often cited as being the prime causes of reflux disease, yet despite this very few research groups are investigating new ways to evaluate this dynamic structure in the gastrointestinal tract[32,34].

Much work has been done in extending manometric measurements enhancing pH measurement techniques to further evaluate the effects of acid in the oesophagus[72] and developing new techniques for measuring bolus transit

both down to the stomach and back up into the oesophagus using intra-luminal impedance[71]. However, none of these techniques have overcome the fundamental question of how the OGJ function can be quantified.

FLIP may provide the answer to this question. In a way trialing the probe in the OGJ was probably the most challenging and perhaps experiments in another sphincteric region would have been more straight-forward where the dynamic activity would be slower. FLIP may have a role in determining biomechanically the root cause of diseases. It can be used for evaluating therapy for example endoscopic and surgical therapy for reflux. It may be possible to use it intra-operatively during Nissen fundoplication, for example where the tension of the wrap is extremely important. It may also be possible to explore other sphincters in the body with FLIP so that the relationship between their biomechanical properties and their relationship to disease can be further defined.

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TOPIC HIGHLIGHT

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Gastric accommodation assessed by ultrasonography

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Abstract

Gastric accommodation is important for the understanding of the pathophysiology in functional dyspepsia and is also relevant for symptom generation in other disorders. The term gastric accommodation has at least three different meanings: The accommodation *process*, the accommodation *reflex*, and the accommodation *response*. The gastric accommodation *process* is a complex phenomenon that describes how the size of the gastric compartment changes in response to a meal. The electronic barostat is considered the gold standard in assessing gastric accommodation. Imaging methods, including MRI, SPECT, and ultrasonography may also be used, particularly in patients who are stress-responsive, e.g. functional dyspepsia patients, as a non-invasive and less stress-inducing method is favourable. Ultrasonography satisfies these criteria as it does not by itself distort the physiological response in stress-responsive individuals.

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Key words: Ultrasonography; Gastric accommodation; Stomach; Barostat; Gastric relaxation; 3D ultrasound

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INTRODUCTION

Gastric accommodation has become a key mechanism for the understanding of the pathophysiology in functional dyspepsia[1,2] and may also be relevant for symptom generation in other disorders[3-6]. Several methods can be used to assess gastric accommodation[7]. The electronic barostat is considered to be the gold standard method for measuring proximal gastric accommodation[8,9]. By applying the barostat, gastric tone is estimated by recording variations in the volume of air within an intragastric bag that is maintained at a constant pressure by the instrument. Accommodation of the proximal stomach is supposed to be reflected in changes of gastric tone. However, the method is invasive, bothersome and affects normal gastric motility[10]. The bag may also alter intragastric distribution and emptying of the meal the direct stimulus imposed by the bag itself on the stomach wall results in increased antral distension[11]. Therefore, non-invasive methods as MRI, SPECT, and ultrasonography are warranted for the study of gastric accommodation.

MRI is a safe and accurate technique to image the entire stomach with detail[12]. After ingestion of a test meal labelled with gadolinium as an MRI marker, images of the gastric region are acquired, typically using a Spin Echo T1-weighted sequence. The MRI technique permits three-dimensional reconstruction of the stomach, enabling total gastric volume measurements during fasting and postprandially. The ratio of these measurements is used to quantify the accommodation response. Single Photon Emission Computed Tomography (SPECT) uses ^{99m}Tc uptake into the gastric mucosa for visualisation and volume estimation of the gastric compartment[13]. Comparison of SPECT data with barostat measurements showed that SPECT is able to detect changes in intragastric volume but unable to detect changes in gastric tone and hence accommodation[14].

GASTRIC ACCOMMODATION

Conceptually, the process of gastric accommodation has been explored for over 100 years, evaluating relaxation of the proximal stomach[15]. Receptive relaxation has been defined as a vagal reflex, which dilates the corpus-fundus region of the stomach in response to swallowing, oesophageal stimulation, or vomitation[16-18]. Adaptive relaxation is a intrinsic vagovagal reflex that enables the stomach to increase its volume markedly without a corresponding increase in pressure[19,20]. Adaptive relaxation enables the stomach to maintain a low balanced pressure and to continuously adapt its volume to its content.

However, the term gastric accommodation is too

complex to embrace in one definition, mainly because it is utilized in at least three different meanings in the literature: The accommodation *process*, the accommodation *reflex*, and the accommodation *response*. In addition, the term is often used in an everyday language expressing that "the stomach accommodates a meal". Similarly, it was also used by Paton and Vane in 1963 to describe gastric "expansion to accommodate the fluid"[21]. The gastric accommodation *process* is a complex phenomenon that describes how the size of the gastric compartment changes in response to a meal[22]. It involves both intra- and extragastric reflexes. The extragastric reflexes comprises both receptive relaxation and intestino-gastric reflexes elicited by balloon distension or nutrient infusion in the duodenum[8,23]. The intragastric reflexes embrace at least adaptive relaxation and relaxation of the proximal stomach induced from antral reflexes[24-26].

Kelly[27] described the accommodation *reflex* when he demonstrated how intragastric pressure was regulated by gastric "accommodation to distension". The accommodation reflex is elicited when continuous volume expansion of the proximal stomach reaches a certain level, thus inducing relaxation of smooth muscles of the gastric wall in order to prevent further increase in pressure. This physiological phenomenon is a reflex arc consisting of a mechanoreceptor in the gastrointestinal wall, a neuro-muscular pathway through ENS with a reflex centre, and a well-defined response in the effective organ. The reflex is probably vagally mediated but local axon reflexes may also be involved[20]. The term accommodation *response* is the effect of the *reflex* or the result of the *process* and this is a precise notion for what we intend to measure with different methods.

IMAGING METHODS

A crucial question is whether imaging methods, as MRI, SPECT or ultrasonography, actually can be compared to the measurements made by the barostat. In our opinion, imaging methods and the invasive barostat method do not measure the same aspects of the gastric accommodation *process*. Thanks to its close contact with the gastric wall the barostat bag adjusts to changes in proximal gastric pressure by changing the intrabag volume. Thus, changes in volume are believed to reflect changes in muscle tone of the wall. However, the quantitative change in volume seen during barostat examinations is only valid during barostat studies using exactly the same equipment and positioning technique. Imaging methods, however, visualize directly the size of the gastric compartments, thus giving an indirect measure of relaxation and contraction. The volume change seen using imaging can thus be explained by additional secretion, air retention or most probable changes in gastric emptying.

The gastric meal accommodation *process* has two components: Passive meal distension of the gastric compartments and active muscle relaxation of the gastric wall. The first component is best measured with imaging methods whereas the barostat is best suited for studying the second component. Imaging methods at this stage, do not distinguish between enlargements of the stomach due to reflex relaxation or due to meal-induced distension; it just measures the totally accommodated volume. Accordingly, it may not be adequate to compare imaging methods to the barostat for validation of gastric accommodation. Gastric accommodation depends on neuromuscular factors and hence it is also a matter of evaluation of the mechanical properties of the stomach. In this sense the barostat merely detects the existence of change in wall tone, but cannot as the imaging methods, provide data on the distribution of the volume and the normal behaviour of the gastric wall. In biomechanics, it is essential to understand the geometry of the organ and the forces and deformation in different directions in order to better understand the active-passive muscle function and mechano-sensation in health and in diseases such as the functional disorders. This warrants 2D and 3D image analysis of the gastric compartments.

The different imaging methods exhibit different spatial and temporal image resolution, and these factors strongly influence accuracy in volume calculation. MRI and in particular SPECT imaging have poor spatial and temporal resolution compared to ultrasonography. Ultrasonography can provide temporal resolution above 100 fps, if necessary, and a spatial resolution on sub-millimetre level. Also, the "stress-factor" of the imaging methods should be considered in this context because dyspeptic patients in general and vagal reflexes in particular are very sensitive to psychological stress. Everyone who has been inside a narrow, noisy MR scanner knows how frightening this can be. Naturally, the stress-factor is also substantially involved in studies using the barostat. Simply because functional disorders are so strongly associated to psychological factors, the examination should be performed in a quiet and relaxing atmosphere with a minimum of distress. Ultrasonography satisfies these criteria as it is non-invasive and does not by itself distort the physiological response in stress-responsive individuals. Moreover, due to gravity playing a central role in the propulsion of gastric content, the study of meal accommodation should preferably be performed in a "natural position" such as sitting in a chair. Therefore, methods that enable patients to be seated have an advantage over methods requiring patients to be in supine position during the examination.

Ultrasonography

Ultrasonography, a clinical method that is widely available, has shown applicability and validity for the study of gastric accommodation[28]. The 2D method developed by Gilja *et al*[29] has been used to study gastric accommodation in functional dyspepsia[1,30], diabetes[4], in patients with reflux oesophagitis[5], in liver cirrhosis[31], and in children with recurrent abdominal pain[6]. This 2D method has also been applied to study pharmacological intervention[32] and the effect of a barostat bag in the stomach[11]. Another advantage with 2D ultrasonography is its clinical applicability; it can easily be performed bedside and repeated numerous times in the same subject. At Haukeland University Hospital we have for 20 years used The Ultrasound Meal Accommodation Test (U-MAT) for the work-up of patients with dyspepsia (Table 1). Our mainstream clinical protocol consists of a standard soup meal (500 mL), ultrasound

Table 1 The ultrasound meal accommodation test

Time	Protocol
Fasting	Ordinary ultrasound examination of the liver, gallbladder, biliary tract, spleen, pancreas, kidneys, and large vessels.
Fasting	Evaluation of symptoms by VAS.
Fasting	Assessment of motility pattern (phase 1-3) by observing the pattern of contractility in the antrum.
Fasting	Measurement of area of the distal stomach (AA).
Fasting	Visualisation of the proximal stomach to explore whether it has content.
Meal ingestion	500 mL of preheated meat soup is ingested in 4 min at a constant speed.
2 min pp.	Measurement of the sagittal area (SA), the oblique frontal diameter (OFD), and the antral area (AA).
5 min pp.	Postprandial symptom evaluation.
10 min pp.	SA, OFD, and AA measurement.
20 min pp.	SA, OFD, and AA measurement.
30 min pp.	SA, OFD, and AA measurement.

The ultrasound meal accommodation test (UMAT) was developed at Haukeland University Hospital on the basis of close interaction between scientific and clinical work in patients with dyspepsia. Before entering the protocol, the patients have been carefully worked-up with history, physical examination, blood tests, testing for H pylori, and upper endoscopy. In some cases, additional examinations are performed to rule out organic causes for their symptoms. The protocol presented here is the mainstream clinical protocol. In scientific studies other elements are often added. A 500 mL liquid meal of commercial meat soup (Toro × clear meat soup, Rieber & Søn A/S, Bergen, Norway) containing 1.8 g protein, 0.9 g bovine fat, and 1.1 g carbohydrate (83.68 kJ) is ingested over a period of 4 min. The soup is preheated and then cooled to 37 ℃ to improve imaging quality by reducing the amount of air bubbles. Psychometric evaluation is also performed.

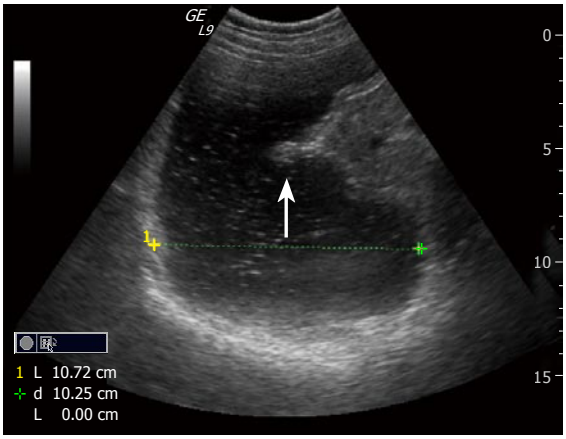


Figure 1 Ultrasonogram showing an oblique frontal section of the proximal stomach in a patient with dyspepsia referred for Ultrasound Meal Accommodation Test (UMAT). The transversal diameter is measured indicating the size of the postprandial proximal stomach and hence indirectly degree of accommodation. Interestingly, one can also observe a gastric contraction (white arrow) in the posterior angular part and progressing towards the antrum.

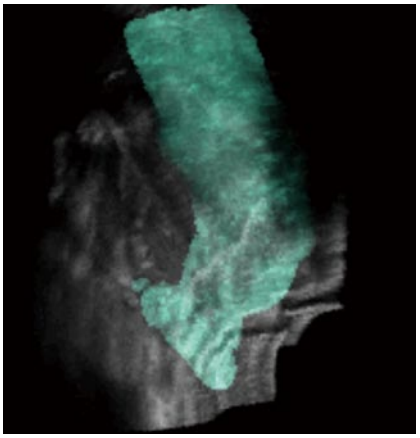


Figure 2 3D image reconstruction based on ultrasound acquisition with a magneto-based position- and orientation measurement system (POM) using GE Logiq 9. The 3D image is overlayed the ordinary gray scale values of ultrasonograms.

scanning of the proximal and distal stomach using predefined scan sections (Figure 1), calculation of size and volumes of the gastric compartments, evaluation of symptoms and psychological assessment. In our experience, ultrasonography used in this context adds valuable clinical information to the management of these patients.

There are few methods that are capable of measuring all parameters of gastric motility simultaneously. However, many authors have demonstrated that ultrasonography can be used to study not only gastric accommodation, but also gastric emptying[33-39], gastric configuration[40-42], intragastric distribution[43], antral motility[44-46] and distensibility[47,48], transpyloric flow[44,49-51] and strain of the gastric wall[42,52,53]. Of the present available methods, we would argue that ultrasonography is the method that has been shown to estimate most parameters of gastric motility, many of these also simultaneously. Furthermore, we claim that ultrasonography has the strongest potential as a clinical tool compared to MRI, SPECT, and scintigraphy, and particularly in patients with functional disorders.

3D ultrasound

For better visualization of the total stomach and improved calculation of gastric volumes, 3D ultrasound imaging of the gastric compartments was developed[43,54,55] It is important that validation of an imaging method to study gastric accommodation also include evaluation of its accuracy and precision in volume estimation. In our lab, we have utilized two different systems for acquisition of 3D ultrasound images and both systems have been validated *in vitro* and *in vivo*. First, we developed a mechanical system with the advantage of an accurate motorized movement of the ultrasound transducer in a tilting fashion. This 3D system demonstrated excellent accuracy and precision *in vitro*[56,57] and good agreement with MRI *in vivo*[58]. Subsequently, we applied a magneto-based system (Bird sensor, Ascension Technology Corp., Burlington, VT, USA) that enabled greater flexibility during scanning and larger volumes like the total gastric compartment to be captured (Figure 2). The Bird system also showed excellent accuracy *in vitro*[43,59-61] and in volume measurement of a thin-walled fluid filled bag in the human stomach the agreement with true volumes was excellent[62]. Accordingly, 3D ultrasonography delivers high resolution, is accurate in volume estimation and is a well-suited tool to monitor volume changes of the stomach during the *process* of accommodation. However, we acknowledge its limitations: impairment of image quality with air in the GI tract, mainly for use with liquid meals, and scanning of very obese subjects (fortunately dyspeptics are not often obese).

Abnormalities of gastric accommodation are a significant finding in several patients groups, not only in patients with functional disorders. Comprehension of gastric geometry, the forces and deformation during meal accommodation, and the active-passive muscle function is important in functional diseases[63]. Of the present available methods, we would argue that ultrasonography is the method that has been shown to estimate most parameters of gastric motility, many of these also simultaneously[64]. Furthermore, we suggest that ultrasonography has the strongest potential as a clinical tool, particularly in patients with functional disorders.

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TOPIC HIGHLIGHT

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Functional findings in irritable bowel syndrome

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Abstract

The pathophysiology of IBS is complex and still incompletely known. Both central and peripheral factors, including psychosocial factors, abnormal GI motility and secretion, and visceral hypersensitivity, are thought to contribute to the symptoms of IBS. Several studies have demonstrated altered GI motor function in IBS patients and the pattern differs between IBS subgroups based on the predominant bowel pattern. Few studies have so far addressed GI secretion in IBS, but there are some evidence supporting altered secretion in the small intestine of IBS patients. Visceral hypersensitivity is currently considered to be perhaps the most important pathophysiological factor in IBS. Importantly, several external and internal factors can modulate visceral sensitivity, as well as GI motility, and enhanced responsiveness within the GI tract to for instance stress and nutrients has been demonstrated in IBS patients. Today IBS is viewed upon as a disorder of dysregulation of the so-called brain-gut axis, involving abnormal function in the enteric, autonomic and/or central nervous systems, with peripheral alterations probably dominating in some patients and disturbed central processing of signals from the periphery in others.

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INTRODUCTION

Irritable bowel syndrome (IBS) is characterized by abdominal pain and/or discomfort related to abnormal bowel habits[1]. It is probably the most common disorder encountered by gastroenterologists[2] and also the most common gastrointestinal disorder seen in primary care[3]. A problem in the management of IBS is that there are no structural or biochemical disease markers for these patients. Therefore, various diagnostic criteria have been used to define IBS in clinical practice and, more importantly, in research settings[1]. In the Western world, IBS appears to affect up to 20% of the population at any given time[4-6], although the prevalence figures vary substantially depending on the definition of IBS[7,8]. Due to its high prevalence and, for many patients, chronic nature and incapacitating symptoms the cost of IBS to society is substantial[9].

Despite being very common, the pathophysiology of IBS is far from understood. IBS has for a long time been considered a disorder of disturbed gastrointestinal motility, although uniform motility patterns in IBS have been hard to define. Moreover, motility findings have been difficult to relate to symptoms, especially abdominal pain[10,11]. Beginning in the early 1970s, but progressing rapidly in the 1990s were theories that visceral hyperalgesia independent of enhanced motility could explain the painful symptoms of IBS[12]. More recently, a more integrated model of brain-gut interactions proposes that the central and enteric nervous systems interact and modulate enhanced motility, abnormal sensation and autonomic reactivity: the brain-gut axis. In context with this model, psychosocial factors, rather than being a cause of IBS, are regarded as contributing to the predisposition, precipitation and perpetuation of IBS symptoms, and affect the clinical outcome[13]. However, despite the increasing knowledge of the pathophysiological and clinical aspects of IBS, there are a substantial number of unresolved issues.

During recent years the interest in finding relevant alterations in patients with IBS in the function of the gastrointestinal tract, as well as in the central and autonomic nervous system has increased. The trend is now that there is less focus on pure gastrointestinal alterations and instead more focus on interactions between exacerbating factors and gastrointestinal function, as well as on interaction between different parts of our nervous system and the gastrointestinal tract. This article will review the literature

on findings in IBS patients supporting disturbed function in the gastrointestinal tract as well as its interaction with the central and autonomic nervous systems.

ALTERED GASTROINTESTINAL MOTILITY

Esophageal and gastric motility

Most studies of motility in functional bowel disorders have focused on the large and small bowel, although there are also reports on disturbed motility in the esophagus and the stomach. Patients with IBS demonstrate, for instance, lower pressure in the lower esophageal sphincter and more esophageal contraction abnormalities than healthy subjects[14]. Furthermore, patients with contraction abnormalities in the esophagus often report symptoms compatible with IBS[15]. There are several studies of gastric emptying in patients with IBS. These have come to somewhat different conclusions, with some of the older studies being unable to detect an abnormality[16-18], whereas some of the more recent studies found abnormal gastric emptying in at least a subset of IBS patients[19-22]. Moreover, the presence of gastroparesis in IBS was found to be related to small bowel dysmotility[23]. In a recent large-scale study, delayed gastric emptying was found in IBS patients with overlapping dyspepsia, but not in those without dyspeptic symptoms[24]. Some studies have also used electrogastrography (EGG) in patients with IBS finding a nice correlation between lack of increased postprandial EGG amplitude and delayed gastric emptying in a subset of IBS patients[25], whereas another study found EGG abnormalities only in IBS patients with concurrent dyspeptic symptoms[26]. The conclusion of these studies seems to be that altered esophageal and gastric motor function is more related to the presence of upper GI symptoms than to IBS per se, even though a significant overlap between upper GI symptoms and IBS exists[27].

Small intestinal motility

The motor patterns of the small bowel in man are rather well characterized[28], but marked inter- and intraindividual variations make interpretation difficult[29,30]. In the late 1970s technological advances made it possible to accurately record small bowel motility in man. Thompson and co-workers, using a radiotelemetry capsule system, reported abnormal small-bowel motility in a patient with "irritable colon"[31], but a follow-up study using a similar recording system failed to confirm this[32]. Subsequent studies have focused on the periodicity of the migrating motor complex (MMC) (i.e. the interval between successive MMC cycles), as well as finding specific motor patterns in IBS and relating these patterns to symptoms. In diarrhea prone subjects, the periodicity of the MMC during daytime has been found to be shorter[33,34], but longer in IBS patients with predominant constipation[35], although these are not consistent findings[36,37]. Increased frequency of clustered activity has been found in some[33,34,38], but not all studies[36,37,39]. Of more interest perhaps, are correlations between symptoms in IBS patients and certain motility patterns, such as clustered activity (e.g. "discrete clustered contractions") and prolonged propagated contractions[33,34], but likewise, these findings have been partly[37] or totally[36] rejected by others. Furthermore, IBS patients have been found to have enhanced perception of physiological intestinal motility, such as the activity front[40]. There also appears to be a relationship between heightened sensitivity to distension of the small intestine and postprandial jejunal motor abnormalities in IBS patients[41]. An increased contraction frequency postprandially has been demonstrated in diarrhea and constipation predominant IBS[42], and in a study by Schmidt et al diarrhea prone IBS patients had more aborally propagated contractions in phase II and postprandially than the controls[37]. The contraction amplitude has, if anything, been shown to be increased in IBS patients with diarrhea[37] and reduced in those with constipation[35,42]. Our group has used high-resolution manometry in the small intestine with closely spaced recording points to evaluate the propagation pattern of individual pressure waves[43]. A study in IBS patients and healthy controls disclosed a higher proportion of duodenal individual pressure waves that propagated in a retrograde direction in IBS, especially in the postprandial phase. Moreover, when combining this high-resolution analysis with more conventional manometry analysis, abnormal findings were found in the majority of IBS patients[38].

Motility disturbances of the small bowel have also been demonstrated indirectly in transit studies, with rather uniform findings of accelerated transit through the small bowel in diarrhea predominant IBS[44,45] and delayed transit in constipation predominant IBS[18,44]. Small intestinal bacterial overgrowth (SIBO) is a condition caused by an abnormal number of bacteria in the small intestine, which is a common complication of severe small intestinal motor dysfunction[46,47]. Recently, provocative studies, using the lactulose hydrogen breath test[48], have proposed that SIBO is present in approximately 80% of IBS patients[49] and that this alteration responds favourably to antibiotic treatment with symptom improvement[50]. Moreover, the presence of SIBO in IBS patients has been proposed to be related to small intestinal motor abnormalities[51]. However, contradictory results exist and the relevance of SIBO in IBS is still open to debate[52-54].

To conclude, there is evidence of disturbed small bowel motility in IBS patients as a group, but no uniform motility pattern has been found and a consistent correlation between motility findings and symptoms has been difficult to demonstrate. Whether the observed motility abnormalities of the small intestine in IBS are due to factors related to the enteric or the central nervous system is not clear, but there is evidence to suggest that both may be true[35,39,55].

Colonic motility

Considerable effort has been made to find a specific abnormality in the myoelectrical and/or motor patterns of colon in IBS. However, a primary role for colonic dysmotility in the pathophysiology of IBS has been hard to demonstrate. Some of these difficulties may be explained by the fact that myoelectrical and motor events from the colon are less predictable and less well studied than corresponding events elsewhere in the gastrointestinal tract[56,57]. In the 1970s Snape and colleagues reported on

abnormal colonic myoelectrical activity in IBS (3 cycle/min pattern)[58,59], but subsequent studies failed to confirm this pattern to be specific for IBS[60,61]. Instead, since this pattern was demonstrated to be equally common among psychoneurotics without gastrointestinal symptoms it was suggested that this abnormality represented a colonic response to stress rather than being related to IBS[62]. Other techniques have focused on colonic spike patterns and demonstrated variations in the incidence of these[63], but whether these findings simply represent a non-specific response to diarrhea is not clear, since appropriate non-IBS controls have not been studied.

Several studies of basal colonic motility with intraluminal pressure recordings have not been able to demonstrate an abnormal pattern in IBS as compared to appropriate controls or differences between IBS patients with different symptomatology[17,64], but this has to some extent been contradicted in recent studies of colonic motility[65,66]. Some of the earlier studies were, however, able to detect frequent sigmoid contractions with high amplitude in association with pain reports from some of the IBS patients studied ("spastic colon"), although no group differences in rectosigmoid motility between IBS patients and controls could be detected[67]. Also, some recent studies have found an increased frequency of high-amplitude propagating contractions (HAPC) in the colon in non-constipated IBS patients[65,68,69], and more interestingly an association between pain episodes and HAPCs[65,70], possibly related to the presence of visceral hypersensitivity in IBS. Somewhat more consistent has been the finding that IBS patients seem to have an exaggerated and prolonged postprandial myoelectrical and motor response than healthy volunteers[17,65,71,72], even though negative studies also exist[66,73].

The normal motor response in the colon after nutrient administration is an increase in phasic motor activity and a progressive increase in tone as well[74]. At least two studies have failed to detect an abnormal colonic tone response after meal intake in IBS patients[66,75]. However, there are some recent data supporting altered gastrointestinal reflex activity in IBS patients, by demonstrating abnormal recto-sigmoid tone response following a low-caloric meal[76], or as an attenuated rectal tone response to colonic distension ("the colorectal tonic reflex")[77,78]. Hopefully these interesting studies will be followed by other investigations evaluating gastrointestinal reflex activity within the gastrointestinal tract, thereby providing new important information regarding neuromuscular function within the gastrointestinal tract in IBS patients. As in the small intestine motility alterations have also been demonstrated using transit studies. Transit through the colon influences the bowel habit of the patient[79,80], and differences exist in colon transit between IBS subgroups based on the predominant bowel habit[44,81].

To conclude, there is some evidence to suggest a disordered colonic motility in IBS, even though no uniform colonic motor pattern of this patient group is evident. Of great relevance to the symptomatology is probably the exaggerated colonic motor response to physiological stimuli such as food and, perhaps also stress and emotions in IBS[61,82], which together with an enhanced visceral sensitivity might be deleterious for these patients. Alterations in gastrointestinal reflex activity in IBS patients seem plausible, but follow-up studies are needed.

ABNORMAL GAS HANDLING

In patients with IBS complaints of "too much gas" causing abdominal pain and bloating is very common, and this has been proposed to be secondary to disordered intestinal motility in combination with an abnormal GI sensitivity, rather than caused by abnormal volume or composition of intestinal gas[83]. However, an abnormal colonic fermentation in IBS has been demonstrated in one study[84], but needs to be repeated by other groups. Of great interest, patients with IBS complaining of bloating has been found to have impaired transit of exogenous gas loads, and the following gas retention reproduced their symptoms[85]. Importantly, this abnormality can be modulated by nutrients[86, 87], physical activity[88] and body posture[89]. In follow-up studies using scintigraphy the small intestine seemed to be responsible for the ineffective gas propulsion in patients complaining of bloating[90], and altered gastrointestinal reflex activity in IBS patients might also be involved in the abnormal gas handling[91].

These findings tell us that IBS patients do not necessarily need to produce more gas in order to have gas related symptoms, but these may instead be due to motor dysfunction, resulting in a transport problem, and an enhanced visceral sensitivity.

ALTERED GASTROINTESTINAL SECRETION

Few studies have so far addressed GI secretion in IBS, partly due to methodological problems in assessing gastrointestinal secretion. However, there are some evidences supporting altered secretion in the small intestine of IBS. An old study demonstrated that patients with IBS have enhanced intestinal secretion in response to perfused bile acids in the ileum, relative to controls[92]. Our group has used potential difference measurements in the small intestine as an indirect measure of secretion in the small intestine[93], and in preliminary studies found an enhanced reactivity of the secretory component of the MMC in the small intestine in diarrhea-predominant IBS and to some extent also in constipation predominant IBS[94]. Ongoing studies will further evaluate the importance of secretory abnormalities in patients with IBS and the relevance for symptoms.

VISCERAL HYPERSENSITIVITY

Since James Ritchie in 1973 demonstrated colonic hyperalgesia by inflating balloons in the sigmoid colon in patients with "the irritable colon syndrome"[95] numerous reports on visceral hypersensitivity in IBS have appeared. Lowered perception thresholds for balloon distension in IBS patients have not only been demonstrated in the rectum[96] and colon[97], but also in the esophagus[98,99], stomach[100] and the small intestine[101,102]. These findings support a generalized enhancement of gastrointestinal sensitivity in IBS patients. However, a recent investigation has implicated that the hypersensitivity seems to be pan-intestinal for

those with multiple sites of their symptoms, whereas it was restricted to a specific organ in those with more localized symptoms[103]. Whether IBS patients have a general hypersensitivity remains debatable since divergent results exist regarding somatic sensitivity[104-106]. Patients with IBS also show an altered viscerosomatic referral pattern as another indicator of disturbed processing of viscerosensory information[107]. However, visceral perception is not abnormal in all patients with IBS[100,108] and there is a considerable overlap in colonic pain thresholds between IBS patients and healthy controls [109]. Moreover, the relationship to the symptom profile of the patient is controversial[108,110-112].

In an attempt to find a biological marker for IBS, Mertz et al evaluated different aspects of rectal perception in a group of IBS patients. They found that 94 percent of patients showed altered rectal perception in the form of lowered thresholds for aversive sensations (discomfort), increased intensity of sensations or altered viscerosomatic referral[96]. Also, a more recent study has come to the same conclusion and proposed that rectal sensitivity testing is useful to confirm the diagnosis of IBS and to discriminate IBS from other causes of abdominal pain[113]. The proposal that altered rectal perception is a biological marker for IBS has, however, been questioned and the importance of psychological factors in visceral perception has been stressed instead[114]. Psychological factors might certainly be of great importance in studies dealing with visceral sensations, and response bias[115] may influence results from balloon distension studies. A different conclusion can be drawn from a study by Corsetti *et al*, who showed that there was no difference between patients and healthy controls in identifying regular balloon inflations mixed with sham distensions, suggesting that the increased frequency of sensations often reported by IBS patients are not due to psychological response bias[116]. On the other hand, Whitehead and co-workers found that thresholds were correlated with psychological measures of anxiety and somatization[117]. However, in that study sexual abuse, reported to be associated with IBS[118], did not contribute significantly to the rectal pain sensitivity, which has been confirmed recently[119]. One explanation for these somewhat discrepant results may be that IBS patients demonstrate two different perceptual alterations: hypersensitivity to rectal distension, which is proposed to be largely due to peripheral alterations, and hypervigilance towards labeling a wide range of visceral stimuli in negatively affective terms, which is more related to psychological alterations[120].

A drawback with many of the studies assessing visceral perception in IBS is that they are made under fasting and non-stimulated conditions, under which patients normally do not report severe symptoms. However, it has been shown that repetitive sigmoid distensions - thought to imitate increased colonic motility after food intake[17], emotions[82] and stress[61] - resulted in hyperalgesia and increased viscerosomatic referral during rectal distensions in IBS patients. These findings were proposed to reflect a central sensitization of the dorsal horn neurons[121]. These data fit well with findings from studies evaluating the effects of rectal distension on spinal transmission of nociceptive signals, using a somatic cutaneomuscular flexion reflex (RIII reflex)[122], where IBS patients have an altered response[123]. Whether this is designated to changes in electrophysiological properties of the spinal nociceptive neurones or failure in the inhibition of nociceptive signals in spinal nerves is yet to be answered.

Patients with IBS often describe a correlation between their symptoms and stress, and they seem to be more susceptible to stressful events of daily life[124]. Using different methodologies, several studies now exist that demonstrate an altered visceral perceptual response in IBS patients relative to control subjects after and during stress[125-127], which might be of relevance for stress-related symptoms in IBS patients. Postprandial worsening of symptoms, as well as adverse reactions to one or more foods is common in IBS patients[128]. The effects of nutrients on colorectal sensitivity have been investigated in healthy volunteers and rectal sensitivity increases after nutrient ingestion[129]. Fat is also the main stimulus for this sensory component of the gastrocolonic response[130]. Recently we demonstrated enhancement of the colonic sensitivity in IBS patients after duodenal lipid administration as compared with healthy controls[75]. This finding has thereafter also been reproduced by other groups assessing the effect of duodenal lipid administration on rectal sensory function[131,132], making it fair to state that patients with IBS seem to have an exaggerated sensory component of the gastrocolonic response.

Visceral hypersensitivity may also be affected by motor characteristics and biomechanical properties of the gut, but studies assessing for instance rectal compliance have come to somewhat inconclusive results[108,133,134]. Moreover, a study using impedance planimetry found no clear evidence of altered biomechanical properties in the rectum in patients with IBS and hypersensitivity[135]. However, more studies assessing the relationship between sensory and motor characteristics and biomechanical properties of the gastrointestinal tract in IBS patients are needed.

To conclude, visceral hypersensitivity is currently considered to be perhaps the most important pathophysiological factor in IBS. Importantly, several external and internal factors can modulate visceral sensitivity. The link between visceral hypersensitivity and specific symptoms in IBS deserves further studies.

DYSREGULATION OF THE BRAIN-GUT AXIS

The brain-gut axis is a model describing bidirectional pathways linking emotional and cognitive centres in the brain with visceral afferent sensation and intestinal function. Several observations have led to the hypothesis of a dysfunctional brain-gut axis in the pathophysiology of IBS[136-138]. Brain imaging techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) have been used to investigate brain response during visceral stimulation with rectal distensions. Despite contradicting results, several studies indicate differences between IBS patients and healthy controls in activation of different pain processing regions such as the anterior cingulate cortex (ACC), thalamus, insula and prefrontal cortex (PFC)[139].

Using PET, Silverman et al initially reported that painful rectal distension in IBS patients resulted in reduced

ACC activity but enhanced activity in the PFC during anticipation of rectal pain suggesting a hypervigilance towards the stimulation and a failure to activate endogenous pain inhibition systems[140]. However, Mertz et al later published an fMRI study showing increased activity in the ACC indicating a normal activation pattern but a heightened sensitivity of the brain-gut axis[141]. Several brain imaging studies have been published thereafter with somewhat divergent results, with reports of increased activity in the insula, PFC and thalamus[142,143], as well as in the cingular cortex[142,144,145], but also decreased activity in the insula, amygdala and striatum[146]. The discrepancies in results are probably explained by factors such as small study populations and pooling of results from heterogeneous groups, as well as the use of different methodologies[147]. It has for instance been demonstrated that male and female IBS patients show differences in activation of brain regions[148,149], just like the activation pattern differs between IBS subgroups based on the predominant bowel habit[150]. The psychological state of the patient is also important since emotional status has been shown to modulate brain activation patterns[151]. Interestingly, changes in some brain regions have also been associated with treatment response in IBS[152]. In addition to brain imaging, IBS patients have also shown different results than healthy controls in studies using electroencephalography (EEG)[153,154] and evoked potentials[155,156].

Central nervous system communication with the gut is mediated through the parasympathetic and sympathetic pathways of the autonomic nervous system (ANS) by modulation of the enteric nervous system. Several studies have reported autonomic dysfunction in IBS[157-161]. The results are inconsistent, but increased sympathetic and decreased parasympathetic activity in IBS patients compared with healthy controls are the most frequently reported differences[162-166]. Again, the contradictory results may partly be due to varying experimental designs with differences in study populations. Anxiety and depression influences autonomic function in IBS[167], and there also seem to be discrepancies between different subgroups of IBS patients[162,165,168]. Recently Tillisch *et al* also showed gender differences in ANS response to visceral stimulus, with more prominent alterations in male IBS patients[169].

Another important system in the communication between the brain and the gut is the hypothalamic-pituitary-adrenal (HPA) axis, with important effects on GI motility, sensation and immune function[170]. Activation of this system takes place in response to both physical and psychological stressors[171]. Since patients with IBS often report stress-related symptoms[124], it has been a logical step to evaluate the HPA axis and its effect on GI function in IBS. Also here results are divergent with investigations reporting attenuated[172], normal[125] and enhanced reactivity[127,173] of the HPA-axis in IBS patients in response to various stressors. Again, different study designs, patient characteristics and other factors might be the explanation for the somewhat heterogeneous results[174].

To conclude, there appears to be alterations along the brain-gut axis and in autonomic nervous function in patients with IBS. However, there are discrepancies between existing studies, and in future studies standardization of the methodology, as well as strict inclusion criteria and evaluation of larger patient groups will enhance our understanding of the interaction between the gut and different parts of our nervous system.

CONCLUSION

The pathophysiology of IBS is complex and still incompletely known. Both central and peripheral factors, including psychosocial factors, abnormal GI motility and secretion, and visceral hypersensitivity, are thought to contribute to the symptoms of IBS. However, in order to be able to find new treatment alternatives for this big patients group more knowledge about the pathophysiology of IBS is needed.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, *Series Editor*

Evaluation of the biliary tract in patients with functional biliary symptoms

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Abstract

The aim of this paper was to describe functional biliary syndromes and methods for evaluation of the biliary tract in these patients. Functional biliary symptoms can be defined as biliary symptoms without demonstrable organic substrate. Two main syndromes exist: Gallbladder dysfunction and sphincter of Oddi dysfunction. The most important investigative tools are cholescintigraphy and endoscopic sphincter of Oddi manometry. In gallbladder dysfunction a scintigraphic gallbladder ejection fraction below 35% can select patients who will benefit from cholecystectomy. Endoscopic sphincter of Oddi manometry is considered the gold standard in sphincter of Oddi dysfunction but recent development in scintigraphic methods is about to change this. Thus, calculation of hilum-to-duodenum transit time and duodenal appearance time on cholescintigraphy have proven useful in these patients. In conclusion, ambient methods can diagnose functional biliary syndromes. However, there are still a number of issues where further knowledge is needed. Probably the next step forward will be in the area of sensory testing and impedance planimetric methods.

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Key words: Functional biliary pain; Scintigraphy; Manometry; Pain; Sphincter of Oddi; Gallbladder

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INTRODUCTION

Evaluation of the biliary tract is of importance in physiological, pathophysiological, and clinical settings. Clinical investigations in patients with well-defined, organic diseases such as gallstones, is outside the scope of this review, where focus is directed towards patients with functional biliary symptoms. Investigations aiming at morphological alterations in the biliary tract are thus not discussed in this paper.

The value of different investigative methods is often fluid: Some methods are used to render probable or document a correlation between symptoms and measurable abnormalities. Others are, or later become, real tools in the diagnostic process of patients with functional biliary symptoms. The methods can roughly be subdivided into two subgroups: Motility investigations, and methods for the evaluations of the sensory conditions. Motility comprises direct measurement of the biliary tract as well as intraluminal transport. A further evaluation of wall characteristics, e.g. by means of impedance planimetry, has, so far, only been conducted in animal experiments[1].

The present review will start with a physiologic background section, followed by a description of patients with functional biliary symptoms and evaluation methods in these patients.

PHYSIOLOGIC BACKGROUND

The function of the biliary tract is to store, concentrate, and transport bile from the liver into the duodenum. In order to accomplish this function the extrabiliary tract is composed of a ductal system, a gallbladder, and a sphincter (Sphincter of Oddi) at the termination of the common bile duct. Previously it was believed that there was an additional sphincteric structure at the junction site of the gallbladder and the cystic duct but studies have failed to prove this supposition. Smooth muscles are present in the gallbladder and in the Sphincter of Oddi (SO). Unlike previous belief, no-or only insignificant-

musculature is seen in the ducts. Active contractility is therefore only possible in the gallbladder and the SO. Bile flow into the duodenum occurs after food intake, and late in the interdigestive phase II of the duodenum[2]. The main initiator of biliary secretion-apart from bile production of the liver-is the release of the peptide cholecystokinin (CCK). This peptide relaxes the SO and contracts the gallbladder. The effect of CCK on the SO is brought about by a stimulatory action on inhibitory neurons. This effect overrides a direct stimulatory action of CCK on the SO muscles[3]. In addition to the CCK effect, a reflex relationship exists between the gallbladder and the SO, i.e. pressure increase within the gallbladder leads to relaxation of the SO (Figure 1)[4,5]. This implies that gallbladder contraction, e.g. after food intake, allows bile to run from the gallbladder into the ductal system, and further into the duodenum through the relaxed SO. Conversely, when the gallbladder is empty, the gallbladder pressure is low, and the SO actively contracts, thus allowing bile to divert into the gallbladder. A similar reflex relation is seen between the common bile duct and the SO[6]. This is important in post-cholecystectomy patients.

The gallbladder receives nerve fibres from the sympathetic and the parasympathetic systems. In addition there are a number of neurones containing peptides, e.g. CCK, vasoactive intestinal polypeptide (VIP), nitric oxide (NO), and motilin. The sympathetic nerves arise at the Th8-9 level and are relayed in the celiac plexus, with second order fibres travelling along the vascular system to the gallbladder. The parasympathetic innervation arises from both the anterior and posterior vagal trunks and reaches the gallbladder in a similar way as the sympathetic fibres, with the exception that branches from the anterior vagal nerve do not pass through the celiac plexus. Vagal activity stimulates gallbladder contraction directly, and enhances the contractile effect of CCK on the gallbladder. Sympathetic activity has the opposite effect on gallbladder motility. The sympathetic system also transmits sensory inputs to the central nervous system leading to pain. Pain is enhanced by inflammation. The ductal system and the sphincter of Oddi (SO) are innervated in a similar way as the gallbladder, with the highest neural density in the SO.

FUNCTIONAL BILIARY PAIN

Biliary pain is mainly characterized according to the symptomatic presentation of gallstone attacks. As in many other gastrointestinal disorders this is not simple, as a variation in symptomatic presentations can be seen in some patients[7]. Nevertheless, according to the existing literature, and the experience of experts, functional biliary pain was defined as absence of demonstrable organic substrate, and[8]: Episodes of severe steady pain located in the epigastrium and right upper quadrant. A number of additional criteria are outlined. One of these is that the pain attack should last at least 30 min. This seems substantiated in patients where the symptoms are derived from the gallbladder. However, if symptoms originate in the SO, this criterion will still await validation. Two types of functional biliary pain syndromes have been described: Gallbladder dysfunction and sphincter of Oddi dysfunction (SOD). The symptomatic presentation seems rather similar, but obviously patients with gallbladder dysfunction still have the gallbladder in situ, whereas SOD dysfunction most often-albeit not unequivocally-is seen in post-cholecystectomy patients.

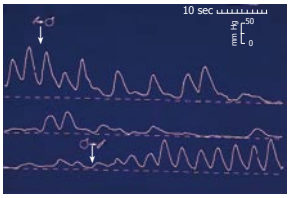


Figure 1 Sphincter of Oddi manometry in a dog during gallbladder distension (upper tracing), and gallbladder emptying (lower tracing). Distension of the gallbladder-mimicking gallbladder contraction -leads to relaxation of the sphincter of Oddi thus permitting free flow of bile into the duodenum. (After reference 5 with permission).

Gallbladder dysfunction

Gallbladder dysfunction denominates a condition where the gallbladder empties insufficiently in a patient with biliary symptoms, without demonstrable organic substrate, such as gallstones. The prevalence of biliary-type pain without gallstones was found to be 2.4%[9]. Other population studies have found prevalences as high as 7.6% in men[10] and 20.3% in women[11]. Per definition patients with gallbladder dysfunction have impaired gallbladder emptying. Later in this paper the methodology is described. The mechanism of pain is rather obscure: Generally it is believed that some kind of functional obstruction at the transition zone between the gallbladder and the cystic duct exists, leading to gallbladder pressure increase. Possibly, a combination with inflammation amplifies sensory firing. Primary hypersensitivity can also not be ruled out and central neuroplastic changes may be responsible for visceral hypersensitivity. Nevertheless, in a randomised controlled investigation[12], it was shown that patients with gallbladder dysfunction would benefit from cholecystectomy. A number of other papers have supported this finding[13,14]. However, there are still a number of critical issues to be addressed[15].

Sphincter of Oddi dysfunction

Sphincter of Oddi dysfunction (SOD) denominates patients with biliary-type pain and dysfunction of the Sphincter of Oddi. The condition is most often seen in post-cholecystectomy patients. The prevalence of symptoms suggesting SO dysfunction is about 1.5% after cholecystectomy, being more frequent in women[16]. SO dysfunction can be subdivided into three groups according to clinical presentation, laboratory results, and ERCP findings[17]: (1) Patients with type I SO dysfunction have biliary-like pain, elevated liver function tests documented on two or more occasions, delayed contrast drainage (more than 45 min, (Figure 2), and a common bile duct diameter equal to or greater than 12 mm at ERCP; (2) Type II patients present with biliary-like pain, and only one or two of the above mentioned criteria; (3) Type III patients only have biliary-like pain, and none of the above mentioned criteria. The mechanism of pain is also rather obscure in this group of patients. From a theoretical point of view SO abnormalities could give rise to pain due to impeded flow, ischemia arising from spastic contractions, and hypersensitivity of the papilla or the common bile duct.

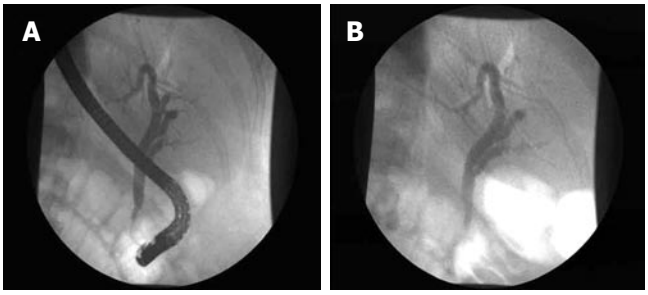


Figure 2 Endoscopic cholangiogram during investigation, and 45 min after contrast injection in a patient with complete contrast retention.

These mechanisms may solely or in combination explain the genesis of pain[18]. In a prospective randomised study it was shown that pain could be alleviated by endoscopic sphincterotomy[17].

EVALUATION METHODS

Quantitative hepatobiliary scintigraphy

Quantitative hepatobiliary scintigraphy (QHBS) is a non-invasive scintigraphic method where it is possible not only to assess the patency of the biliary system but also to measure the rate of bile flow (Figure 3). 99mTc-N-2,6-dimethylphenylcarbamoylmethyliminodiacetic acid (99mTc-EHIDA) and numerous of its analogues, such as 2, 6-diisopropyliminodiacetic acid (DISIDA) and 2, 4, 6-trimethyl-3-bromoiminodiacetic acid (mebrofenin) are the tracers most widely used for hepatobiliary imaging. After (iv) injection, these 99mTc agents bind loosely to serum proteins and are then rapidly taken up by the hepatocytes and excreted into the bile. This uptake mechanism is similar to that of bilirubin, and hyperbilirubinaemia therefore has an inhibitory effect. After excretion by the hepatocytes, the bile continues to flow into the bile canaliculi, the bile ducts and the gallbladder, and then empties into the duodenum through the SO.

Patients should be fasted for a minimum of 4 h prior to the investigation. The gamma-camera is positioned over the right upper abdomen of the supine patient. After an iv bolus injection of 140-180 MBq 99mTc-IDA derivative, gamma-camera images are acquired on a computer (1 frame/min) for 90-120 min. If needed, analogue images can also be obtained every 10 min for the first hour, with delayed images if required (first image 500 000 counts, and then subsequent images for the same length of time). The analogue pictures should be examined for liver radiotracer uptake, size, shape and homogeneity. Under normal circumstances the liver uptake of 99mTc-IDA compounds is rapid, with little if any tracer in the blood pool by 5 min. In most normal fasting subjects, radioactivity appears in the gallbladder (GB) and in the small bowel by 1 h after its administration. The extent of visualisation of the hepatic ducts is variable but the left duct tends to be more prominent than the right. In patients without extrahepatic

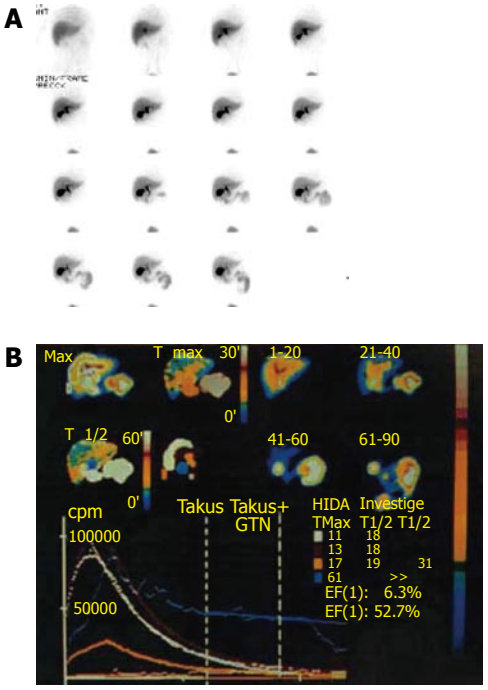


Figure 3 Hepatobiliary scintigraphy. **A:** Pictures at different time windows; **B:** Time activity curves are shown for different regions of interest.

bile duct obstruction, the level of radioactivity of the common bile duct (CBD) reaches its maximum before 45 min. Excretion of radioactivity into the duodenum occurs by 60 min in 81% of patients with the GB *in situ*. In the remaining 19% of patients without bowel activity at 60 min, duodenal visualisation is always present after exogenous CCK administration[19].

Gallbladder phase imaging

The GB contraction after consumption of a meal or administration of an exogenous CCK analogue (CCK octapeptide or caerulein) can also be measured by means of QHBS. These data can be acquired in a separate study between 60 and 90 min after the initial isotope administration. QHBS combined with administration of a meal or an exogenous CCK analogue is a validated and accurate method for the measurement of GB motility, with satisfactory reproducibility of both parametric variables and emptying patterns. The method of measuring GB is to place a region of interest (ROI) over the GB with a ROI over the liver background (adjacent to the GB), then to generate a time-activity curve for the GB and to calculate the GB ejection fraction (EF) via the following formula: EF% = (GBmax-GBmin)/GBmax × 100. The degree of GB emptying is CCK dose-dependent, and therefore the mode of administration has a critical effect on the net GB contraction. The GB EF increases as the dose of CCK is increased from 0.5 to 3.3 ng/kg per minute. Then begins to decrease when larger doses are infused, especially when CCK is given as a rapid iv bolus. A large non-physiological dose produces a low EF in association with abdominal pain, even in normal subjects. It has become clear that the GB EFs obtained depend on the procedure used but a

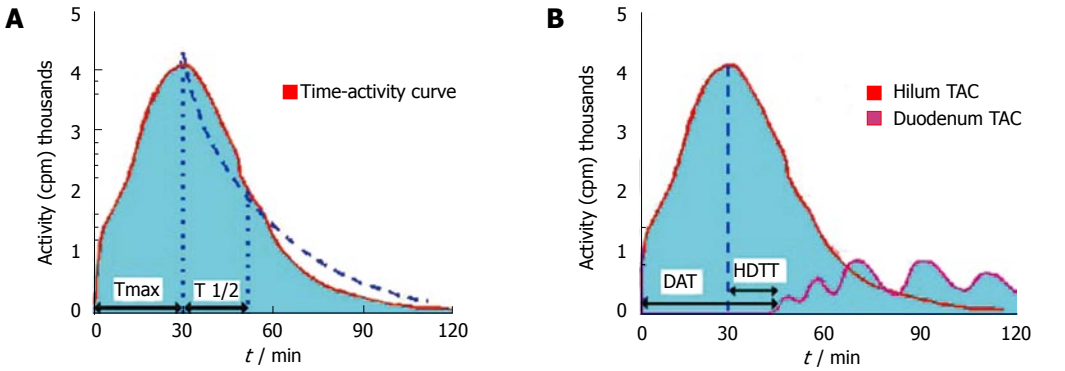


Figure 4 Hepatobiliary scintigraphic activity curves of regions of interest in sphincter of Oddi dysfunction.

GB EF over 35%[12,20,21] can be regarded as indicative of a normal GB function if it is evoked by a total infusion dose of 10 ng/kg CCK, administered during 3-10 min.

Scintigraphic features of gallbladder dysfunction

In clinical practice, the distinctive feature of a GB dysfunction is impaired GB emptying. A GB EF under 35% is a sensitive test for the identification of patients with chronic cholecystitis or GB dysfunction[12,20,21]. However, there is a considerable overlap in EF values[15]. Many of the patients experience a reproduction of biliary pain during CCK infusion. Administration of nitro-glycerine before CCK infusion can abolish biliary pain and normalise the GBEF in a subset of patients with gallbladder dysfunction.

Scintigraphic features of sphincter of Oddi dysfunction

The scintigraphic signs in SOD depend upon the presence or absence of a GB in situ, albeit the latter is rare. In patients with SOD and GB in situ, an intact GB serves as a low-pressure reservoir by compensating the increased bile flow that is diverted into it by the tight and hypertonic SO. Therefore, the CBD does not dilate and the rate of emptying of the isotope from the CBD is usually in the normal range. After CCK administration to patients with a functional SOD, the SO relaxes (except in SOD patients with a paradoxical response to CCK, which is a relatively rare phenomenon) and, if the GB function is intact, the GB EF remains normal. In cholecystectomized subjects with a normal liver function, the flow of the bile from the liver to the duodenum is mainly regulated by the motor activity of the SO. HIDA compounds, which are taken up and secreted by the liver into the bile, can therefore be used as markers to measure the rate of bile flow and indirectly to assess the patency of the SO. In asymptomatic cholecystectomized patients with a normal SO function, HIDA excretion and transit from the liver into the duodenum is rapid, and the time-activity curves are exponential, exhibiting only minor patient-to-patient variations[19]. In cholecystectomized patients with SOD however, cholescintigraphy reveals a prominent bile stasis within the intra- and extrahepatic bile ducts, and a marked delay in the transpapillary isotope transit[22-24]. Retention of a 99mTc-IDA tracer after 120 min in the visually prominent bile ducts on static images of the planar HBS was noted to be a good predictor of an abnormal biliary drainage. Quantitative cholescintigraphic parameters may afford valuable numerical information on the bile flow impairment in the presence of a SOD, thereby increasing the sensitivity (Figure 4). Unfortunately, it is not clear, which scintigraphic variable is optimal to express SOD. We recently compared the sensitivities of these parameters in cholecystectomized patients with SOD diagnosed by manometry, which showed that HDTT was the best predictor of SOD, followed by DAT, and then the $T_{1/2}$ parameters of the hepatic hilum and CBD Madacsy). The quantitative parameters of the CBD seem to be relatively insensitive, which is probably due to the disturbing influence of other factors, such as the CBD diameter and the duodenal motility phase during the study. In fact, we found that the SO basal pressure relative to the CBD pressure exhibited the best correlation with the QHBS parameters. CCK administration increases the hepatic bile secretion and relaxes the SO; it therefore washes out the bile stasis from the ducts if there is no anatomic obstruction. A differentiation between a fixed anatomic obstruction (organic SO stenosis, bile duct stricture or stones, *etc.*) and a functional obstruction (SO spasm) can also be made by amyl nitrate or nitro-glycerine administration during QHBS. In order to measure SO relaxation after the administration of a smooth muscle relaxant in patients with SOD, after the baseline evaluation of the QHBS parameters, at 60 min amyl nitrate was administered and the $T_{1/2}$ parameter of the CBD was recalculated. A reversible biliary obstruction on the QHBS (an obstructive pattern relieved after amyl nitrate) indicates a functional SOD, i.e. an SO spasm, whereas a persistent accumulation of the isotope in the biliary tree (during the baseline condition and after amyl nitrate) suggests a fixed organic obstruction[25]. A differentiation between stenosis and dyskinesia of the SO may be important from a clinical point of view, and it might modify the therapeutic strategy, since endoscopic sphincterotomy is of great benefit in patients with a persistently elevated basal SO pressure and SO stenosis. To summarise, QHBS may be regarded as a useful method for study of the bile flow dynamics in patients with postcholecystectomy syndrome and suspected SOD. QHBS may be applied not only as a simple and non-invasive screening test before SO manometry but also as a reliable diagnostic method during the evaluation and follow-up of patients with suspected SOD.

Ultrasonography

US has become a widely used method for visualisation

of the abdominal organs. As it is a non-invasive method without radiation exposure, it has also become a popular method for the investigation of motility disorders of the GB and the SO. US methods are relatively cheap and widely available and they also afford excellent reproducibility and sensitivity. However, their diagnostic accuracy is appreciably investigator-dependent and also influenced by the different forms of methodology.

Ultrasonographic measurement of GB contraction

The GB contraction induced by exogenous CCK or CCK analogue (caerulein) administration can be measured by means of either US or QHBS, but methods involving QHBS are currently regarded as the gold standard. QHBS offers the advantage over US of allowing simultaneous visualisation of the bile ducts, together with non-geometrical measurement of the GB contraction. However, the lack of radiation exposure is a favourable feature of the US methods, because it can be repeated several times in the same patient, which is extremely important in motility studies if normal physiology is investigated. US is also able to determine the approximate volume of the GB, which may be of some importance in the pathophysiology of GB motility. Different formulae are known for calculation of the GB volume. The most widely used is the sum of cylinders method, or Dodd' s formula. With this method, the GB volume (GBV) can be calculated if one multiplies the three axes together and then by Pi/6[26]. The GB EF is calculated from the fasting and post-stimulation GBVs via the well-known formula: GBEF (%) = GBVmax - GBVmin/GBVmax. The refilling of the GB is another characteristic feature of GB motility, which can be calculated as a percentage of the ratio between the GBV in the most contracted state and at the maximum after refilling. The two main types of stimuli, which can be used to evoke GB contraction, are food intake and administration of CCK or a CCK analogue.

Ultrasonographic bile duct imaging

Bile duct imaging with US is more difficult than visualisation of the GB. Most investigators use the right anterior oblique view to visualise the common hepatic duct within the porta hepatis, where the surrounding liver parenchyma gives a good acoustic window. Visualisation of the pancreatic duct in the body of the pancreas may be problematic in a number of patients, because the presence of gas in the stomach and in the intestines above it means that an acoustic window is not available. Moreover, exact determination of the pancreatic duct diameter necessitates its measurement in two different part of the pancreas. The upper limit of normal diameter of the common hepatic duct (also termed hepatocholedochus, because it is not possible to differentiate between common hepatic duct and CBD by US) is less than 8-10 mm, and of the pancreatic duct is less than 2-3 mm.

Ultrasonographic assessment of sphincter of Oddi dysfunction

Ultrasonography has been reported as a method for diagnosing SO dysfunction. The authors measured the bile duct diameter before and after CCK infusion or ingestion of a fatty meal[27,28]. However, these methods have not gained acceptance, probably because of low reproducibility.

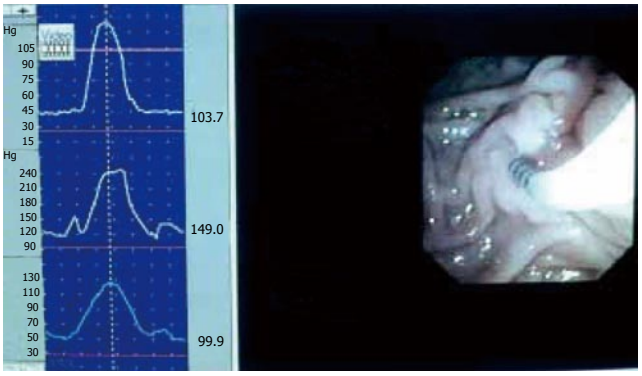


Figure 5 Combined video- and manometric recording in the sphincter of Oddi during endoscopy.

Sphincter of Oddi manometry

SO manometry can be done during endoscopic retrograde cholangio-pancreatographic (ERCP) investigation. A triple lumen catheter with sideholes spaced 2 mm apart is introduced into the common bile duct, and then withdrawn until positioned in the SO segment. The catheter is slowly perfused from a hydraulic capillary infusion pump, and external transducers are connected with a computer for direct reading of the manometric tracing. Additionally the readings are stored for later analysis[29,30]. The SO is a high-pressure zone with an elevated baseline and superimposed phasic contractions. The readings may suffer from periodic superimposed artefact patterns. Usually these are easily recognised, but simultaneous endoscopic videorecording may be helpful in separating SO activity from artefact phenomenon (Figure 5)[31]. The parameters measured are baseline pressure and phasic wave amplitude, duration, frequency, and propagation mode. Baseline pressure is measured with the duodenal pressure as zero reference. The amplitude is measured with baseline pressure as zero reference. When evaluating the propagation modality, proximal and distal readings are compared.

Normal SO manometry

In healthy subjects the baseline pressure is around 5-15 mmHg, phasic wave amplitude 100-140 mmHg, wave duration 4-6 s, and frequency 3-6 waves per minute. Most contractions are coordinated, and the direction of contractions is mostly antegrade, with only 5%-20% being retrograde[32]. After cholecystectomy no significant alterations are seen in patients who no longer have symptoms[33,34].

Abnormal SO manometry

SO manometric abnormalities comprise elevated baseline pressure, increased phasic wave amplitude, frequency, and disordered propagation mode (Figure 6, Figure 7). It has been suggested to define SO abnormality as a baseline

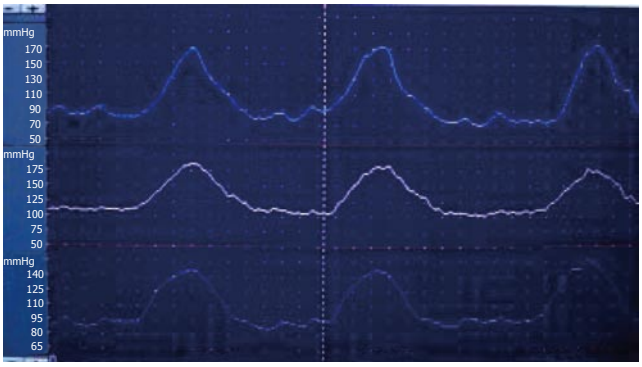


Figure 6 Endoscopic sphincter of Oddi manometry in a patient with elevated baseline pressure.

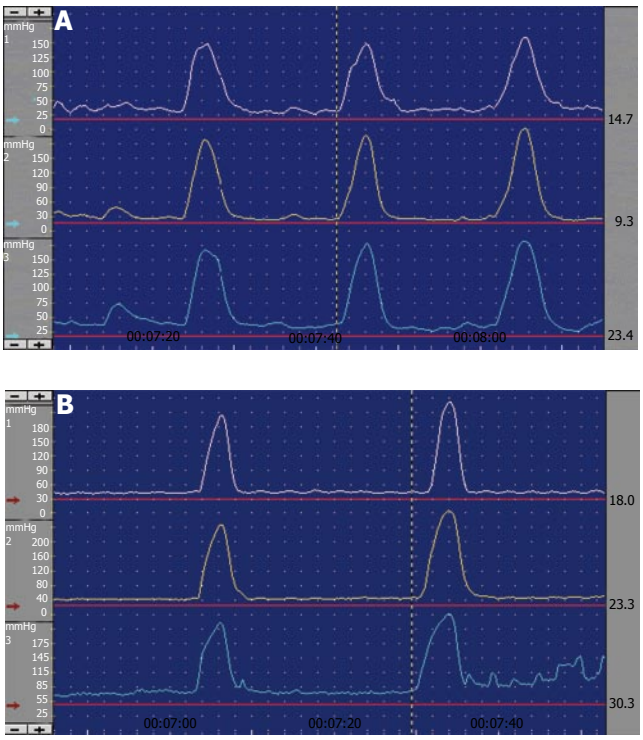


Figure 7 Endoscopic sphincter of Oddi manometry. **A**: Normal antegrade contractions; **B**: Abnormal retrograde waves.

pressure of more than 40 mmHg, phasic amplitude above 350 mmHg, frequency of more than 7 waves per minute; and a pattern of more than 50% retrograde waves[8]. The clinical importance of these abnormalities is not clear, except for elevated baseline pressure, where it was shown in a randomised study that patients with this abnormality improved clinically after endoscopic sphincterotomy[17].

Pain studies

The fact that there are still "holes" in our understanding of patients with functional biliary pain has led investigators to focus on sensory mechanisms. Clinically, it has been observed that some patients develop pain upon manipulation with the papilla (tender papilla), or during intraductal contrast injection. A further step forward was taken with the demonstration of duodenal-specific visceral hyperalgesia in post-cholecystectomy patients with persistent abdominal pain[35]. Stawowy *et al*[36-38] has investigated the referred pain area in patients with uncomplicated gallstones and acute cholecystitis before and after cholecystectomy. Although hypersensitivity was found in both groups of patients, the hypothesized prolonged neuroplastic changes, as a possible explanation of post-cholecystectomy syndrome could not be proven. However, in the future it will be of importance to conduct studies in patients with post-cholecystectomy syndrome and also in patients with suspected gallbladder dysfunction.

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TOPIC HIGHLIGHT

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Upper gastrointestinal sensory-motor dysfunction in diabetes mellitus

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Abstract

Gastrointestinal (GI) sensory-motor abnormalities are common in patients with diabetes mellitus and may involve any part of the GI tract. Abnormalities are frequently sub-clinical, and fortunately only rarely do severe and life-threatening problems occur. The pathogenesis of abnormal upper GI sensory-motor function in diabetes is incompletely understood and is most likely multi-factorial of origin. Diabetic autonomic neuropathy as well as acute suboptimal control of diabetes has been shown to impair GI motor and sensory function. Morphological and biomechanical remodeling of the GI wall develops during the duration of diabetes, and may contribute to motor and sensory dysfunction. In this review sensory and motility disorders of the upper GI tract in diabetes is discussed; and the morphological changes and biomechanical remodeling related to the sensory-motor dysfunction is also addressed.

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Key words: Diabetes; Esophagus; Stomach; Intestine; Motility; Pain; Diabetic neuropathy; Hyperglycemia; Remodeling

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disease requiring lifelong medical attention in order to limit the development of potentially devastating late complications and to manage them if they occur. In the USA, the per capita cost of healthcare in 2002 was $13 243 for patients with diabetes and $2560 for patients without diabetes[1]. Gastrointestinal (GI) disorders are common in diabetic patients[2,3]. As many as 75% of patients attending DM clinics report significant GI symptoms[2]. The entire GI tract from the esophagus to the anorectal region may be affected. Common complaints include dysphasia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting and diarrhea. The symptoms may be severe and substantially decrease quality of life. The pathogenesis of the GI abnormalities is complex of nature, multi-factorial (motor dysfunction, autonomic neuropathy, glycemic control, psychological factors, *etc.*) and is not well understood[4]. A number of abnormal conditions have been described in different segments of the GI tract in patients with diabetic autonomic neuropathy (DAN): esophagus (dysmotility), stomach (dysmotility, delayed emptying) and small and large bowel (dysmotility, delayed transit, bacterial overgrowth and diarrhea)[4]. Only a few studies have addressed the visceral sensory function in DM[5-7] and have demonstrated abnormalities in perception thresholds, vagal tonus and evoked brain potentials in patients with DAN. This indicates that DM related neuronal changes may be located both in the peripheral and in the central nervous system (CNS). As mentioned the entire GI tract may be involved in DM, but our review describes the upper GI tract only.

Many studies have demonstrated prominent morphological changes of the small intestine and esophagus in DM[8-11]. Lately, several studies have described biomechanical remodeling as well as morphological remodeling in experimental diabetic rats[12-18]. Recently, Frøkjær *et al*[19] have shown that both the neuronal function of the contractile system as well as the structural apparatus of the GI tract may be affected in patients with longstanding DM and DAN. Therefore we suggest that

along with DAN and glycemic control, the structural and biomechanical changes may play important roles in the symptomatology of GI abnormalities in long-standing DM. The future management of diabetic patients with GI symptoms may develop accordingly.

SYMPTOMS FROM THE UPPER GI TRACT IN DIABETES MELLITUS

Motor and sensory abnormalities in DM may affect the entire GI tract or part hereof, and the perceived symptoms may originate from one or several parts of the gut, such as the esophagus, stomach and small intestine[20,21]. The prevalence of upper GI symptoms is high in both insulin dependent DM (IDDM) and non-insulin dependent DM (NIDDM)[22-28]. The symptoms relating to the esophagus, stomach and small intestine are as follows: (1) Esophagus: Heartburn, dysphagia and chest pain[37]; (2) Stomach: weight loss and abdominal pain[38]; (3) Small intestine: Diarrhea, discomfort, pain and pseudo-obstruction. Most symptoms are non-specific of nature and may relate to other GI disorders not necessarily related to DM. Therefore, when dealing with GI symptoms, some specific issues need to be addressed. Chest pain may relate to reflux or esophageal motor disorders, but ischemic heart disease and other causes of non-cardiac chest pain are also possible causes and must be excluded. Dysphagia, the most characteristic symptom of impaired esophageal transit, may be caused by motility disorders of the esophagus. It is very important, however, to emphasize that dysphagia is more often caused by mechanical obstruction such as tumors and peptic stenosis than by motility disorders. A number of other incidental conditions must be excluded when diabetic gastroparesis is suspected: gastric outlet obstruction caused by tumors and ulcer disease; metabolic abnormalities such as diabetic ketoacidosis or uremia and side effects of pharmacotherapy. A gastroscopy and biochemical screening followed by physical examination are obligatory requisites to reach the correct diagnosis. The symptoms of diabetic gastroparesis tend to increase in intensity and frequency over the duration of diabetes in patients affected. Most often symptoms are vague and nonspecific such as early satiety, slight abdominal discomfort and perhaps bloating, and fortunately more rarely do nausea and vomiting develop. When nausea and vomiting becomes continuous hospitalization is needed to control glucose homeostasis, electrolytes and fluid substitution is needed. This is a potential life threatening situation. In the long run diabetic gastroparesis may be accompanied by poor diabetic control, weight loss and diminished quality of life partly due to frequent hospital contacts. The symptoms may last for days to months, and do often occur in cycles with symptom free intervals[29,30]. Diabetic patients frequently report abdominal pain and this may be the only symptom of diabetic gastroparesis; however, abdominal pain can also be seen in diabetic ketoacidosis and severe metabolic acidosis[31]. These conditions are followed by other symptoms as well and therefore distinguishable from gastroparesis. Diabetic patients with thoracic polyradiculopathy, a rare condition, may also suffer from abdominal pain[32]. Diarrhea in DM patients may be induced by a number of factors. These may include food composition, abnormal intestinal motility, small intestinal bacterial overgrowth, excessive loss of bile acids, pancreatic insufficiency and more[33]. Abnormal small intestinal motility (rapid or delayed transit) is a frequent condition in the diabetic patients as described below. Rapid transit may induce an increase in intra-luminal contents that reach the caecum, whereas delayed transit may cause bacterial overgrowth, both potentially resulting in diabetic diarrhea. Bacterial overgrowth has been reported in up to 40% of diabetic patients with diarrhea[34,35]. Celiac disease is overrepresented in IDDM and a cause of severe diarrhea to be excluded when dealing with diabetic diarrhea[36]. In its most fulminate form, diabetic diarrhea is a devastating and horrible condition for the person affected, sometimes resulting in catastrophical nightly soiling in bed and an uncontrollable condition during daytime. Most often, fortunately, this condition is self limiting and symptoms are more manageable.

SENSORY DYSFUNCTION

Although both the afferent and efferent nerves are affected in DM, the data related to the sensory dysfunction of the GI tract are sparse compared with those relating to the motor dysfunction of the upper GI tract. Elevation of perception thresholds to esophageal electrical stimulation has been observed in patients with DAN and different severity of GI symptoms[7]. Increased vagal tonus and abnormal evoked brain potentials to mechanical and electrical stimulation of the esophagus has also been shown[5,6]. Rayner *et al* performed isovolumetric and isobaric distensions of the proximal stomach in ten randomly selected patients with IDDM[39]. They demonstrated that the perception of gastric distension during euglycemia was increased compared with healthy controls. To study mechanisms behind postprandial symptoms in patients with diabetes, the gastric accommodation of the meal was assessed by abdominal ultrasound[40]. In DM patients, a large proximal stomach was associated with perception of fullness and a large antrum was associated with perception of pain after a meal. More recently, Frøkjær *et al*[41] used a multimodal stimulation device (Figure 1) to investigate the visceral sensitivity to mechanical, thermal and electrical stimulation in the esophagus and duodenum in IDDM patients with DAN and GI symptoms. This study demonstrated that the patients had decreased sensitivity to the stimulations of the esophagus and duodenum. This indicates that the affection of the sensory nerves is widespread in the GI tract. As the multimodal approach is thought to stimulate the mucosa, submucosa and muscle layers differentially, the disease seems to be generalized to nerves in all layers of the gut.

MOTILITY DISORDERS

Long ago, it was known that abnormal motility of the GI tract occurred during the development of DM[42]. So far many studies have demonstrated that DM patients have slow transit and abnormal motility. The most frequent

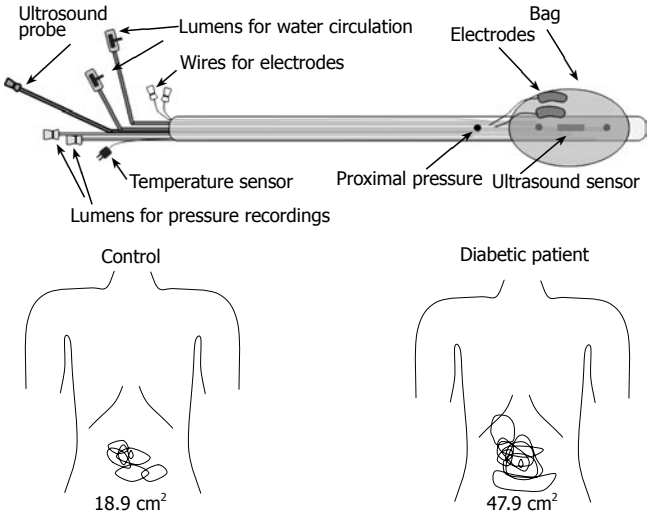


Figure 1 Top: The probe design allowing mechanical, heat and electrical stimulation of both the oesophagus and duodenum. The centre of the bag contains the sensor of the ultrasound probe imaging the oesophagus and duodenum during mechanical distension. The thermal stimuli were given by a pump system, which re-circulates water at 60 ℃ through two channels in the probe. The electrodes allowed electrical stimulation delivered directly to the mucosal surface. Bottom: The somatic referred pain areas to mechanical distension of the duodenum for the controls (left) and diabetic patients (right). The areas were larger for the diabetic patients.

motility disorders of upper GI tract are shown in Table 1.

Upper GI transit disorders in DM

Esophagus: Impaired esophageal transit has been reported both in IDDM and NIDDM patients[43-47]. The esophageal transit appears to be delayed in about 50% of patients with long-standing DM[48]. The retarded esophageal transit in the DM usually reflects either peristaltic failure or focal low-amplitude pressure waves[49].

Stomach: Several animal studies reported a slowing gastric emptying in IDDM and NIDDM rats[50-55], whereas other studies on IDDM and NIDDM animals demonstrated that gastric emptying increased[56-59]. Using radionuclide measuring techniques it has been demonstrated that gastric empting of solid, or liquid meals was abnormally slow in 30%-50% of patients with long-standing IDDM and NIDDM[60,61]. The gastroparesis in DM has been known clinically for more than 50 years[29]. It is not surprising that the gastric emptying delay in DM is related to both slow transit with increased retention of food in the proximal and distal stomach[62,63], and abnormal motility of the gastric wall[64].

Small intestine: Delayed and rapid transit in the small intestine was observed in animal diabetic models[65,66,67,68]. El-Salhy reported that the GI transit was rapid in non-obese diabetic mice[67] and was slower in obese diabetic mice[68]. Anjaneyulu and Ramarao[69] reported an increase in intestinal transit and a decrease in intestinal tone due to increased cholinergic and decreased beta-adrenergic receptor activities in DM rats. Slow small intestinal transit in DM patients have been documented using breath hydrogen appearance time after the ingestion of lactulose[70,71], by using radiopaque markers[72,73] and by use of metal-detector test[74]. On the other hand, Keshavarzian and Iber[75] investigated intestinal transit in IDDM patients after the ingestion of both liquid and solid meals and showed abnormal fast intestinal transit in their sample of diabetic patients. Nguyen *et al*[76] used intraluminal multiple impedance measurements to identify the postprandial duodenal chyme transport in patients with long standing IDDM. They demonstrated that the patients had disturbed propulsive chyme transport through the duodenum and the duodenal chyme clearance activity was decreased.

Table 1 Motility disorders of upper GI tract in DM

Organ	Motility disorder
Esophagus	Amplitude[88,89] and number[46,90] of peristaltic contractions↑
	Number of spontaneous and non-propagated contractions↑[45]
	Amplitude of lower esophageal sphincter pressure↓[88]
	Multi-peaked contractions[78,79]
Stomach	Antral IMMC↓[87]
	Post-prandial antral activity and the number of antral contractions↓[91]
	Pyloric dysmotility[92]
Small intestine	Frequency and amplitude of the antropyloroduodenal contractions↑↓[93]
	Duration of MMC cycle↑[85]
	Early recurrence of the MMC and clusters of contractile activity[94]

The transit disorders can occur in any region of the gut and in every stage of diabetes[74], and can affect each other. Since transit of food through the esophagus is relative fast, the gastric emptying rate is the major determinant of the food delivery to the small intestine. The relationship between esophageal transit and the rate of gastric empting appears to be poor[43] and the gastroparesis is often associated with the intestinal transit delay in DM[74,77].

Abnormal patterns of upper GI motility in DM

Esophagus: Esophageal manometric abnormalities occur in over 50% of patients with DM (see Table 1)[37]. The reduced amplitude of lower esophageal sphincter pressure is in accordance with the increased prevalence of gastro-esophageal reflux disease in DM. More recently the evoked esophageal contractile activity to standardized bag distension was assessed using a specialized ultrasound-based probe by Frøkjær *et al*[19]. A balloon-like bag was positioned 10 cm above the lower esophageal sphincter and inflated. It was demonstrated, both at the bag and 6 cm proximally, that the distension induced hyperreactivity and impaired the coordination of the contractions in the diabetic patients.

Stomach: Motility disorders of the fundus and pylorus have been demonstrated in the diabetic animals[80-83]. In the human studies, it is recognized that disordered gastric contractile activity as assessed by manometry and gastric emptying occurs frequently in DM[84,85]. The motility disorders may include three aspects: Inter-digestive migrating motor complex (IMMC), amplitude and frequency of contractions, and pyloric dysfunction (Table 1).

Small intestine: Camilleri and Malagelada reported that

small intestinal motility was abnormal in about 80% patients of long-standing DM with delayed gastric emptying[86]. Both postprandial and fasting small intestinal dysmotility in the DM was reported (Table 1). Dooley *et al*[87] studied fasting GI motility by manometry for a mean of 210 min in a group of 12 NIDDM patients with diarrhea and DAN. The patients showed grossly disordered motility. The migrating motor complex (MMC) disorders reflect the prolongation of phase II without change in the duration of phase I and III. The results of studies on postprandial motility at the level of the small intestine are inconsistent. However, abnormal motility patterns were observed in the diabetic subjects[85].

Table 2 Acute hyperglycemia and upper GI disorders

Motility disorder	Sensory disorder
Peristaltic wave duration[98]↑	Amplitude of the cortical evoked brain potentials to moderate esophageal distension↑[98]
Peristaltic velocity in the distal part of the esophagus↓[103]	Perception during proximal gastric distension in healthy subjects both in the fasted state and during intra-duodenal lipid infusion↑[95]
Pressure of the low esophageal sphincter↓[103]	Influencing the postprandial fullness in IDDM patients↑[105]
Gastric emptying of both solids and nutrient liquids↓[104]	
Motility index and propagation of duodenal and jejunal waves↓[84]	
Cycle length of inter-digestive motor activity in the fasted state↓[56]	
Small intestinal transit↓[71]	
Proximal duodenal pressure waves↓[104]	

PATHOPHYSIOLOGY OF GI MOTILITY AND SENSORY DYSFUNCTION IN DM

Hyperglycemia

Disordered GI function in DM has been attributed to irreversible DAN but it is now clear that acute high blood glucose concentration *per se* have a major reversible influence on upper-GI tract motility and sensory function. An animal study demonstrated that the correction of hyperglycemia to euglycemic levels restores the delayed transit[65]. Several studies in both healthy subjects and DM patients have shown that the GI motor function is impaired during acute hyperglycemia (Table 2)[84,95-97]. Marked acute hyperglycemia affects the motility in every region of the GI tract[98]. This may indicate that cholinergic activity is affected during hyperglycemia. Hyperglycemia may also affect the perception of sensations arising from the GI tract (Table 2). However, much of the data have been observational, and there is relatively little information relating to potential mechanisms by which these effects are mediated. Because both stimulatory and inhibitory effects occur during the hyperglycemia, the effects of glucose are likely to be mediated by neural or humoral mechanisms, rather than a direct effect on the smooth muscle of the GI tract. The secretion of pancreatic polypeptide, which is under vagal cholinergic control, is diminished during acute hyperglycemia in healthy control subjects[99]. In healthy volunteers, plasma concentrations of motilin are less during hyperglycemia when compared with euglycemia[100]. Considering the effects of systemic changes in blood glucose concentrations, animal studies have revealed the presence of glucose-responsive neurons in the central nervous system, which may modify vagal efferent activity[101]. Neurons responsive to glucose have recently been identified in the rat small intestine[102], but their response to systemic rather than luminal glucose is unclear. Much work is required to elucidate the neural, humoral, and cellular mechanisms by which systemic glucose levels affects GI motility and sensation.

Peripheral and central neuronal changes

DAN is seen as a major factor in the pathogenesis of disordered GI motor and sensory functions in DM[106,107]. Although GI manifestations related to DAN are diverse, many GI complications of DM seem to be related to DAN[19,48,72,108-110].

Histological findings: The best-characterized signs of damage to the autonomic nervous system during DM are morphological animal studies[111-117]. The number of myelinated axons in the vagosympathetic trunk is decreased in diabetic rats[118]. In the GI tract, many changes of nerves and ganglia were observed i.e. (1) dystrophic axonopathy[119]; (2) degeneration of mesenteric nerves and ganglia[111]; (3) number of vasoactive intestinal peptide (VIP)-IR neurons in myenteric ganglia[112]; (4) relative volume density in myenteric plexus[120]; (5) number of adrenergic and serotonergic neurons[111]. In addition, the surrounding tissue is also often disturbed. There is a thickening of the endothelium[121,122], which may sensitize axons to damage from increased pressure or decreased oxygen and glucose availability. It is thus possible that axonal damage may be secondary to disorders in tissue surrounding the neurons. Nitric oxide (NO) is a key neurotransmitter in the regulation of GI motor function[123,124]. In rodents with streptozotocin (STZ)-induced diabetes, NO synthase expression in the gastric myenteric neurons is diminished[125] and associated with delayed gastric emptying. The results of morphologic studies of the vagal nerves in diabetic patients are less consistent. One study demonstrated a decrease in unmyelinated axons in the abdominal vagus, another study found no abnormalities[126,127].

Clinical findings: One study has shown that abnormal esophageal motility is more frequent in the diabetic patients with evidence of DAN than in those without[37]. The presence of abnormal gastroesophageal reflux in diabetic patients has also been associated with the existence of DAN[128]. Increased cholinergic tone may relate to multipeaked waves in the diabetic esophagus in these patients[78]. Gastric emptying largely depends on vagus nerve function, which can be severely disrupted in DM[60]. Studies using cardiac autonomic nerve function tests to assess the involvement of the autonomic nerve system in diabetic patients have indicated that the prevalence and severity of dysmotility of the small intestine is substantially greater in DM patients with DAN compared to patients with normal autonomic function[129]. Diarrhea is evident in 20% of diabetic patients, particularly those with known DAN[130,131]. Altogether, abnormal upper GI motility in DM has been associated to DAN. However, the question arises as to whether neuropathic changes in the intramural GI plex-

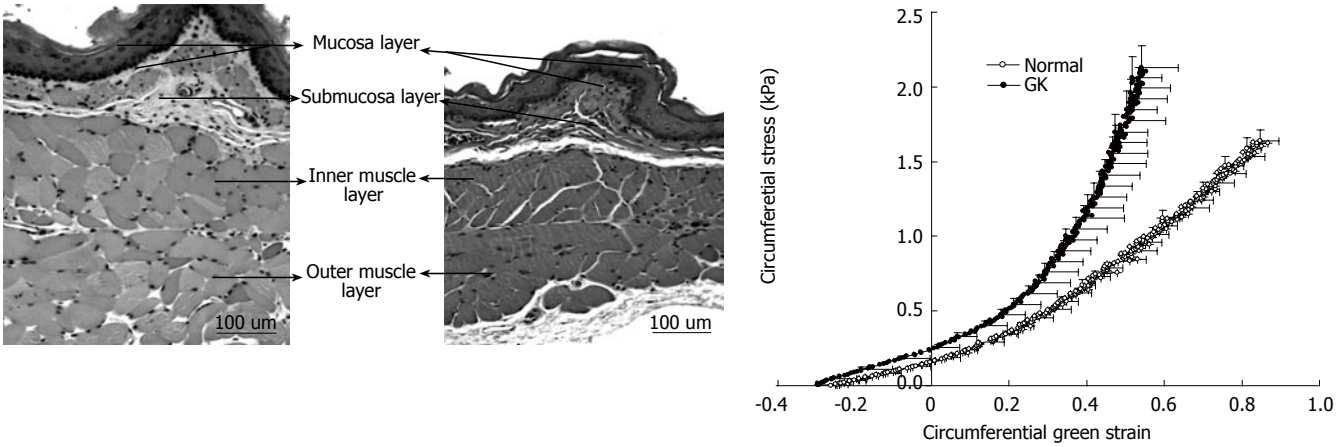


Figure 2 Microphotos of esophageal layer thickness in GK rats (left) and normal rats (middle). The muscle layer was biggest in the GK esophagus. Correspondingly the circumferential stress-strain curves of diabetic and non-diabetic esophageal wall showed in the right. The curve in the GK rats was shifted to the left, indicating that the esophageal wall stiffness increased.

uses, the extrinsic neuronal pathways or in the central nervous system are primary or secondary. In a recent study, Frøkjær *et al* found hyperreactivity and impaired coordination to bag distension of the esophagus in IDDM patients, which indicates that a neuronal dysfunction is responsible for the hyperreactivity[19]. This dysfunction can theoretically be addressed to the mechano-sensitive afferent fibers, inter-neurons or efferent fibers located in the esophageal wall. However, impaired extra-intestinal pathways can potentially also affect the motor response. In another study, Frøkjær *et al* demonstrated that IDDM patients had decreased sensitivity to the stimulations of the esophagus and duodenum[41]. This was accompanied by an increase in somatic referred pain areas to the gut stimulation (Figure 1). As the referred pain is a result of convergence between visceral and somatic afferents on central neurons, the findings are indirect evidence for central hyperexcitability and neuroplastic changes. Altogether, this indicate that the neuronal changes can be located both peripherally (receptors, nerve fibers, ganglions) and in the CNS. Sensory nerve dysfunction in the gut may explain why diabetic patients with severe GI motility disorders often do not suffer from the GI symptoms expected from the abnormal motility and motor function. On the another hand a large subgroup of patients with longstanding DM suffers from severe GI symptoms[2,26,27]. This overall hyperalgesia/hypersensitivity is in contrast to the peripheral autonomic afferent neuropathy that most likely impairs the perception from the GI tract. The mismatch between hypo-and hypersensitivity can likely be explained by an impaired balance between peripheral and central neuronal changes. Hence, the impaired function of the peripheral afferent nerves is likely counterbalanced (or even overruled) by increased central (spinal and/or brain) neuronal excitability and this balance may determine the symptoms in individual patients.

It is likely that different pathophysiological mechanisms contribute to the peripheral and central neuronal changes in DM, including metabolic alterations, microvascular changes, and inflammatory changes[132]. Hypoxia, hyperglycemia, and increased oxidative stress in DM contribute directly and indirectly to Schwann cell dysfunction[133]. This will result in impaired paranodal barrier function, damaged myelin, reduced antioxidative capacity, and decreased neurotrophic support for axons. Furthermore, the direct effects of prolonged hyperglycemia through the glycation on nervous tissue are also important in the development of DAN[134-136].

Morphological and biomechanical remodeling

Although the hyperglycemia and neuropathy seem to be the main mechanisms to the motor-sensory dysfunction in the upper GI tract in DM, the question remains whether the disordered motor and sensory functions of the GI tract are only due to the neuronal changes and dysfunction or if primary diabetes-induced structural and biomechanical changes in the GI tract also play a role? Data on the biomechanical properties are crucial for the understanding of the motor function of the GI tract because, (1) peristaltic motion that propels the food through the GI tract is a result of interaction of the passive and active tissue forces and the hydrodynamic forces in the food bolus and (2) remodeling of the mechanical properties reflects the changes in the tissue structure that determine a specific motor dysfunction. Therefore, the morphological and biomechanical remodeling of the GI wall may also be an important factor in the pathogenesis to the GI motor-sensory dysfunction in the diabetic patients.

Esophageal remodeling: Many studies have shown that DM causes morphological changes and biomechanical remodeling in the esophagus. Yang *et al*[13] in the *in vitro* study on the STZ-induced diabetic rat esophagus found that the wall thickness and cross-sectional wall area increased after the induction of diabetes. Twenty-eight days after the diabetic esophagus became stiffer both in shear and in the longitudinal direction. The esophageal remodeling was also found in NIDDM rat esophagus[18]. In the diabetic rats the opening angle and residual strain distribution in the outer surface of the wall decreased, the collagen fraction in the mucosa-submucosa layer and the passive circumferential stiffness of the esophageal wall increased (Figure 2). Furthermore, the esophageal weight per length, wall thickness and cross-sectional wall area increased in the NIDDM rats.

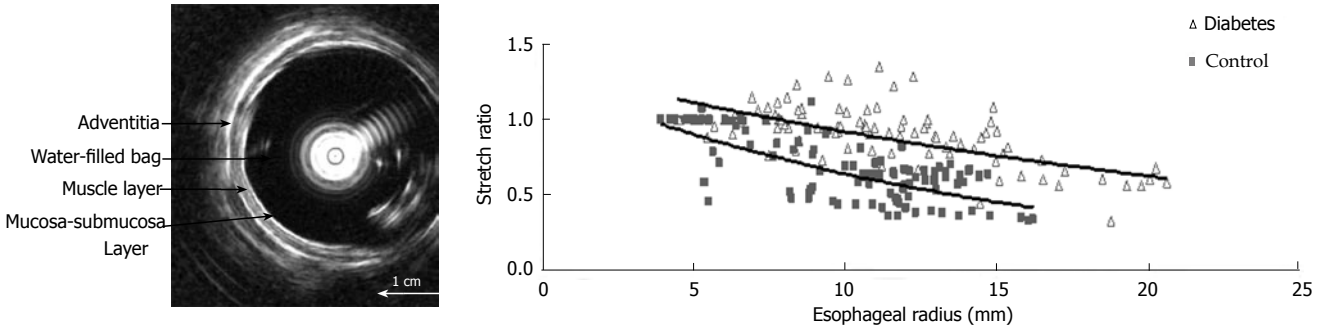


Figure 3 Left: Cross-sectional ultrasound image of the distended distal esophagus allows identification of the esophageal layers, i.e. mucosa-submucosa, muscle and adventitia layers. The white round shadow in the center is caused by the intraluminal ultrasound probe. Right: The distension-induced change in longitudinal stretch ratio is illustrated as function of the esophageal radius. The curves were obtained during smooth muscle relaxation with butylscopolamine. Exponential trend lines of the patients and controls are shown. The shortening during distension was clearly reduced in the diabetic patients.

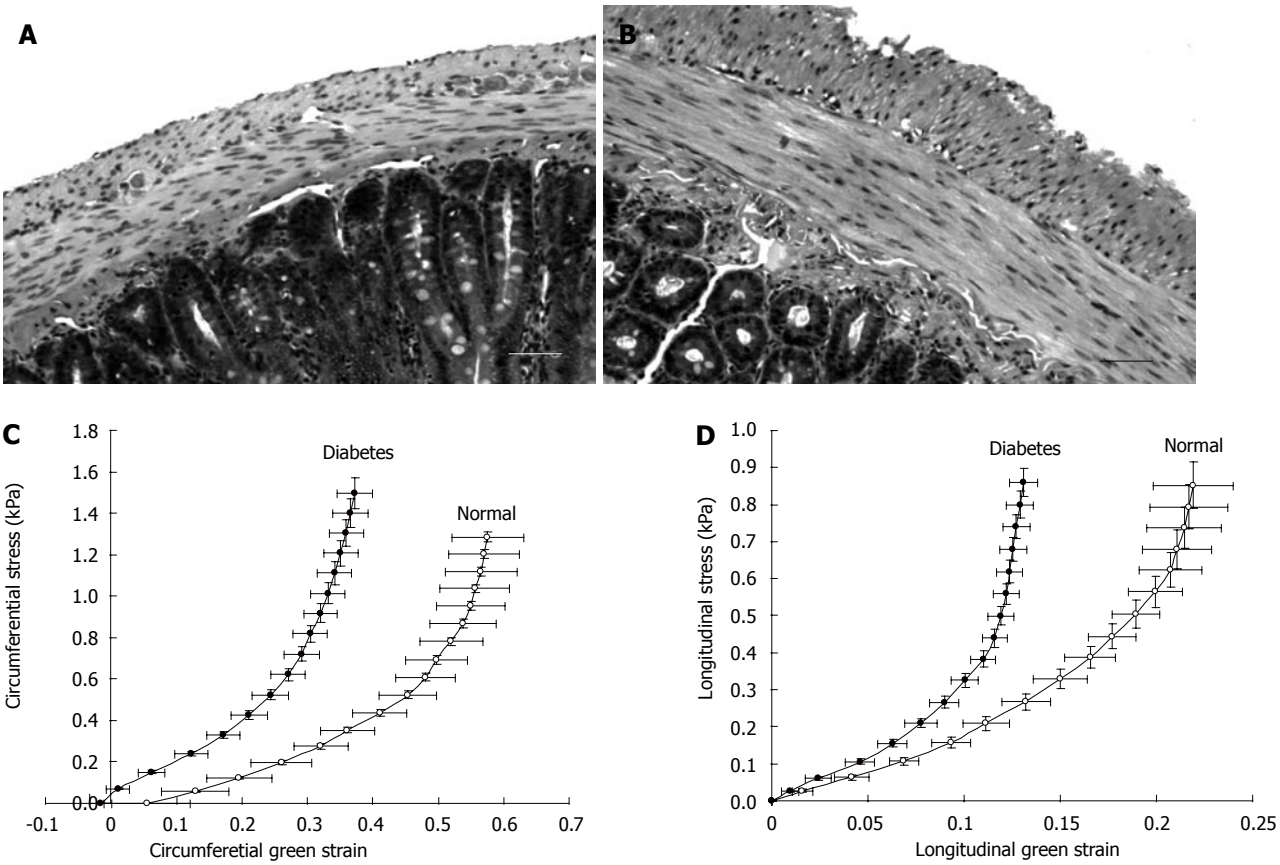


Figure 4 The micro-photographs show the normal (**A**) and 4 wk STZ-induced diabetic (**B**) duodenal histological sections. It clearly demonstrates that the muscle and submucosa layers in the diabetic duodenum became much thicker than in the normal duodenum. The bar is 100 μm. Correspondingly, the circumferential (**C**) and longitudinal (**D**) stress-strain relation curves of the duodenum in the STZ-induced diabetic rats shifted to the left indicating the wall became stiffer.

One previous human study reported that among six cases of diffuse muscular hypertrophy of the esophagus, four cases were associated with DM[137]. Recently, Frøkjær *et al*[19] in an human study found an increase in esophageal wall thickness and altered deformation to the distension with reduced longitudinal shortening and the radial stretch in IDDM patients (Figure 3).

Stomach remodeling: A morphological study in DM rats demonstrated that the gastric mucosa thickness increased in DM rats compared with controls[138]. Remodeling of the interstitial cells network of Cajal in the stomach were found both in animals and humans with DM[139-141]. A histopathological study of the human stomach in DM patients with severe gastroparesis showed prominent collagenization and smooth muscle atrophy of the muscle layer[142]. Regarding the biomechanical remodeling of the stomach only one report by Liao *et al* is available[17]. The rat stomach was distended *in vitro*. Gastric compliance, the surface tension, and circumferential and longitudinal deformation-pressure curves were calculated based on three-dimensional ultrasound reconstructions of non-diabetic and diabetic stomach models. In experimental DM, gastric compliance was lowered both in the non-glandular stomach (proximal part) and the whole stomach (Figure 4). Fur-

Table 3 Morphological and biomechanical changes of small intestine in DM

Morphological change	Biomechanical change
Intestinal weight, length, and weight per unit length↑	Opening angle and residual strain in duodenum↓
Surface area of mucosa↑	Opening angle and residual strain in jejunum and ileum↑
Number of goblet cells per villus↑	Circumferential stiffness of the intestinal wall↑
Smooth muscle mass↑	Longitudinal stiffness of the intestinal wall↑
Different layer thickness↑	Stress relaxation of small intestine↓
Proliferating cell nuclear antigen (PCNA) ↑	
interstitial cells of Cajal volume↓	

thermore, the circumferential stiffness in the non-glandular part increased. The structural changes of the stomach due to DM may together with the sensory and motor nerve dysfunction contribute to the delayed gastric emptying and the symptoms in diabetic patients.

Small intestinal remodeling: Many human[2,11,143,144] and animal [2,3,10,145-148] studies have shown that DM causes morphological alterations in the small intestine (Table 3). However, only few data exist relating to biomechanical remodeling of the small intestine in DM[12]. Recently we did a series of studies on the morphological and biomechanical remodeling of small intestine in the STZ-induced diabetic rats[14-16]. The major findings in our studies are summarized in Table 3 and briefly showed that the stiffness of the diabetic intestinal wall increased in a time-dependent manner (Figure 5). The viscoelastic behavior of intestinal wall also changed during the development of diabetes (Table 3). The stress-strain distribution and viscoelastic behavior mainly reflects the elastic properties of intestine. The changes in elastic properties reflect the structural remodeling of the intestinal wall during the diabetic development.

Advanced glycation end products (AGEs) produced during the development of DM likely relate to the morphological changes and biomechanical remodeling of the GI tract in DM. AGEs are generated by the sequential non-enzymatic glycation of protein amino groups and by oxidation reaction[149]. The accumulation of AGEs in tissues will alter the structure and function of matrix proteins[150]. Studies on DM and ageing show that AGEs are causing cross-linking of collagen molecules responsible for basement membrane thickening and loss of matrix elasticity[151-153]. AGEs may contribute to the diabetic GI morphological and biomechanical remodeling by at least two major mechanisms. The first is receptor-independent alteration of the extracellular matrix architecture by non-enzymatic glycation and the formation of protein cross-links. The second mechanism is receptor-dependent and consists of modulation of cellular functions through ligation of specific cell surface receptors[154-156]. Sanchez SS and coworkers have demonstrated that the expression of small intestinal extracellular matrix proteins have changed in STZ-induced diabetic rats[152]. Therefore, the nonenzymatic glycation of the GI tissue induced by long-term hyperglycemia seems to be an important mechanism behind the GI wall remodeling in DM.

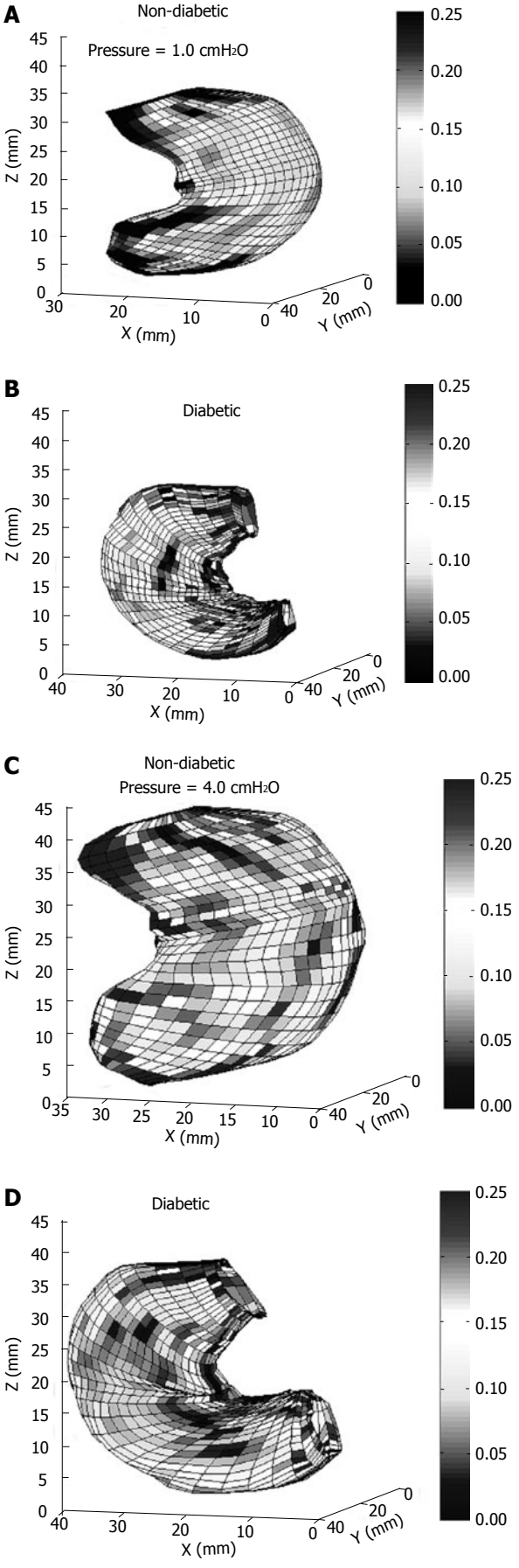


Figure 5 The circumferential curvature at the distension pressure of 1.0 (**A**,**B**) and 4.0 (**C**,**D**) cmH2O in the non-diabetic (**A**,**C**) and the diabetic stomach (**B**,**D**). The circumferential deformation of the stomach in the non-diabetic rats was significantly bigger than that in the diabetic Goto-Kakizaki (GK) rats, indicating the stiffness of the diabetic gastric wall increased in the diabetic GK rats.

Implication of GI remodeling on mechanosensory assessments: Mechanosensation is of fundamental importance for the GI function. The mechanosensitive nerve endings exist extensively in the GI tract where they serve a critical role in homeostasis. Several mechanoreceptor-like structures have been identified such as intraganglionic laminar nerve endings (IGLE) and intramuscular arrays (IMA) in the GI tract[157-164]. The mechanosensitive afferents in the intrinsic and extrinsic pathways were described as low-, wide-dynamic- or high-threshold tension-receptors[165]. Therefore, the GI tract structure as well as the tension, stress and strain distribution in the wall is important for the GI sensory and motor function. The GI wall structure or deformation changes in the DM will alter the relative positions of the mechanosensitive afferents (zero setting of the mechanosensitive afferents)[166]. The biomechanical remodeling in the DM such as alterations of residual strain and stress distribution and increase the wall stiffness will alter the tension and stress distribution of the mechanosensitive afferents. As results, the perception and motility of the GI tract will change as well. Hence, the morphological changes and biomechanical remodeling of GI tract in the DM is likely to affect the function of mechanosensitive afferents in the GI wall and further affect the motor and sensory function. However, so far the data are sparse about the association between the morphological and biomechanical remodeling of GI tract and the motor-sensory dysfunction in the DM[19,41]. The multimodal stimulations have proven accurate and reliable in the assessment of visceral sensation in several studies[167-169]. The geometry data of GI tract can be obtained by impedance and cross-sectional imaging such as ultrasound[170-172]. Combined with pressure recordings, biomechanical parameters such as tension, stress and strain can be obtained and correlated to the symptoms and mechanosensory data in the DM subjects[41]. Therefore, combined studies the GI motor-sensory dysfunction and morphological and biomechanical remodeling in the diabetic GI tract will improve our understanding about the pathophysiology of GI disorders in the DM patients. In the future, animal studies are needed to investigate the passive and active biomechanical and mechanosensory properties of the GI tract related to DM. The experiments should simulate the physiological conditions both *in vivo* and *in vitro* in normal and diabetic animals. The identification of mechanosensitive afferents and enteric nerve responses to the different stimuli will obviously be beneficial to understand the mechanisms of GI motor-sensory dysfunction in the DM.

In conclusion, GI symptoms are frequent in the diabetic patients and are associated with sensory-motor abnormalities, such as impaired perception and motility of the GI tract. The pathogenesis of abnormal GI sensory-motor function in DM is clearly multi-factorial. DAN seems to be the major factor in the pathogenesis of disordered GI motor and sensory functions in DM. Hyperglycemia has also been shown to impair GI motor and sensory function. Furthermore, the morphological changes and biomechanical remodeling of the GI wall may compromise the GI motor function and affect the function of the mechanosensitive afferents in the GI wall. Studies of the relation between the GI motor-sensory dysfunction, morphological changes and biomechanical remodeling in the diabetic GI tract may shed of more light to understand the mechanism of GI motor-sensory dysfunction in the diabetic patients. This knowledge may prove to be valuable in the development of new treatment strategies, which can also be evaluated with the developed methods. New treatments that may be based on the present knowledge and methods are: (1) neurostimulation of afferent visceral nerves, i.e. gastric electric stimulation, (2) agents which can break down already formed glycation end product protein-protein cross-links, and (3) modulation of the central nervous system excitability by drugs.

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TOPIC HIGHLIGHT

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Morphology and motor function of the gastrointestinal tract examined with endosonography

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Abstract

Endosonography is a useful tool for studying the morphology and motor function of the gastrointestinal tract. Intraluminal ultrasonography is the common denomination of ultrasound examinations using intracorporal transducers which are inserted into the GI tract. Thus, the visceral wall and adjacent structures can be imaged in detail. This review describes the usefulness of endosonography in gastroenterology, in particular with respect to studies of the biomechanical and motor function of the gastrointestinal tract. New techniques such as 3-D EUS, elastography and strain rate imaging are discussed.

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Key words: Intraluminal ultrasonography; Gastrointestinal tract; Imaging; Biomechanics

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INTRODUCTION

Disorders of the gastrointestinal (GI) tract may be examined using endoscopy, X-ray, radionuclide studies, electromyography, magnetic resonance imaging (MRI) and manometry. Specific analysis of structural changes occurring within the GI wall is, however, difficult to study by these techniques. Intraluminal ultrasonography (endosonography (ES), endoscopic ultrasonography (EUS) is the common denomination of ultrasound examinations using intracorporal transducers which are inserted into the GI tract. Thus, the visceral wall and adjacent structures can be imaged in detail[1-3]. Acoustic coupling is usually achieved by filling water into the lumen or into a balloon around the ultrasound transducer. Alternatively, the ultrasound probe can be applied directly on the endoluminal surface.

Endosonography is currently mostly performed in the upper GI tract and rectum. However, endosonographic imaging of the colon and distal ileum as well as intraductal ultrasonography (IDUS) of biliary and pancreatic ducts are frequently performed with miniprobes. Dedicated echocolonoscopes are also available, but these have so far gained limited clinical application.

ENDOSONOGRAPHIC INSTRUMENTS

Echoendoscopes combine endoscopy with integrated ultrasound transducers. These instruments can be classified both according to the type of endoscope and the US system. Echoendoscopes may have radial or curvilinear array US transducers using frequencies between 7.5 and 30 MHz. These systems are being continuously improved. The possibility to combine echoendoscopes with conventional ultrasound machines has allowed the use of different ultrasound modalities like B-mode, M-mode and Doppler technology. Ultrasound elastography is a new method with the potential of separating normal from pathological tissue. Recently, elastography and contrast enhanced ultrasound have been applied also in endosonographic imaging[4]. US miniprobes have a diameter which allows the probe to be inserted through the biopsy channel of a conventional endoscope or a suitable catheter (Figure 1). Currently, available US miniprobes comprise mechanical sector, electronic phased arrays, or single crystal linear compound systems operating with frequencies between 12 and 30 MHz. Systems that combine linear compound scanning with mechanical sector scanning or M-mode registration are also available making it possible to switch between these modalities.

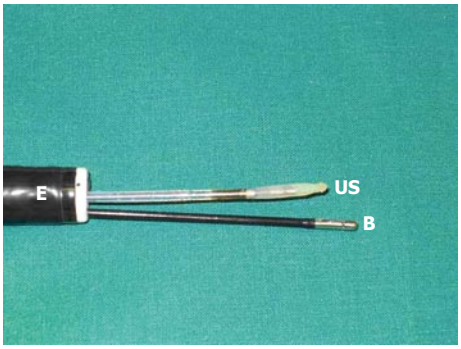


Figure 1 An endoscope with two channels. A miniature ultrasound transducer (US) and a biopsy forceps (B) are inserted through the channels.

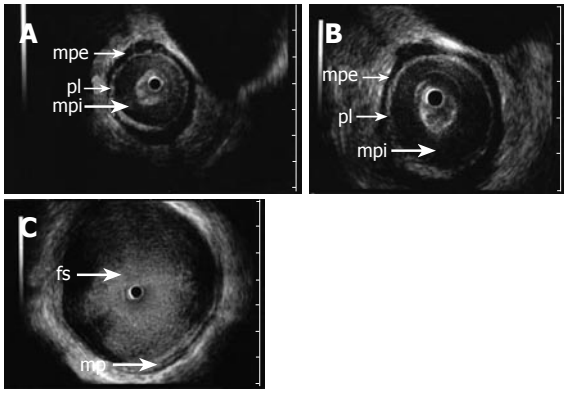


Figure 2 ES images (**A** and **B**) obtained with a 15 MHz miniprobe in a patient with achalasia showing the longitudinal, outer layer of the proper muscle (mpe) separated from a thickened circular, inner layer (mpi) by an echogenic layer corresponding to the localization of the plexus myentericus (pl). **C**: shows a dilated esophageal lumen with food content (fs). A normal aspect of the proper muscle is seen.

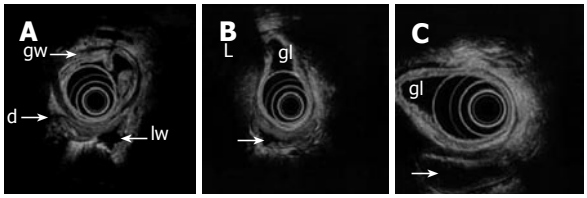


Figure 3 Endosonography with an echoendoscope (12 MHz frequency is used) placed in the gastric lumen (gl) showing the layers of the gastric wall (gw) and GI loops outside the stomach. Lumen is partly collapsed (d), partly waterfilled (arrows). Motility and contractions of native GI loops may be studied. High frequency miniprobes are less suitable for this purpose due to higher frequencies and small transducer diameter.

The small size and high frequencies of miniature probes reduce the size of the US image and the penetration depth of the US beam into the tissue as compared to echoendoscopes. However, small diameter probes can be passed into stenotic areas not traversable with conventional echoendoscopes. Doppler sonography may provide important information about vascular flow and separate vessels from other echo-poor structures. Some echoendoscopes have integrated Doppler technology and Doppler miniprobes for transendoscopic use have also been developed[5]. Other ultrasound systems designed for use within the GI tract do not have an optical system. Transrectal probes have been used for imaging the prostate, bladder, uterus, and rectum. These probes are either linear array or mechanical sector.

ENDOSONOGRAPHIC ASSESSMENT OF GI MOTILITY AND BIOMECHANICS

In recent years ultrasound has been applied in studies of the biomechanical function of the GI tract. In this sense biomechanics should be understood in a much broader sense than the term motility. Motility considers active properties such as peristaltic contractions whereas biomechanics deals with active and passive forces and deformation of the tissue. Biomechanical alterations and abnormal motility in the GI tract is found in several diseases which may be localized in the organ itself or related to the nearest surroundings or to systemic diseases. Luminal manometry has so far been the most widely used method to study GI tract physiology. However, despite the development of high-resolution manometry systems there are still problems in the investigation of GI motor function. The strength of a contraction may vary considerably, thus giving more or less reliable manometric registrations. Furthermore, respiration, aortic pulsation and cardiac contractions may affect the recordings.

The presence of probes in the GI lumen has an influence on physiology and biomechanical studies should therefore ideally be examined without intraluminal devices. ES miniprobes combine the advantages of high ultrasound frequencies with small transducers, allowing detailed images of GI wall layers to be obtained with less influence on the GI lumen than the larger echoendoscopes. Hence, these probes have been regarded suitable for motility studies of the GI tract, especially when applied in lumina with small diameter, like the esophagus (Figure 2). However, echoendoscopes can also be used for examination of motility if positioned in wider lumina like the stomach. Since echoendoscopes generally utilize lower ultrasound frequencies and larger ultrasound probes they can also be used to study motility of neighbouring GI structures (Figure 3). Endosonographic real time B-mode imaging alone or in combination with other methods, as manometry and electromyography, have been used for studying correlation between the pressure and electric potential changes and structural changes in the GI wall. Correct interpretation of ultrasound images of the GI wall is crucial when applying this technique for detailed examination of physiological characteristics. The GI wall consists of mucosa, submucosa, muscularis propria and adventitia/serosa. Muscularis propria has an inner circular and an outer longitudinal layer that is separated by the myenteric plexus.

The normal GI wall is imaged by ultrasonography as a layered structure consisting of 3-9 layers depending on the thickness and structure of different layers, ultrasound frequency, and applied pressure on the wall[6,7]. In most cases, a five-layer structure of the wall can be demonstrated, as determined by the histological layers and the appearance of interface echoes (Figure 2, Figure 4). When the visceral wall is imaged with an endoluminal transducer, the interface between the luminal contents and

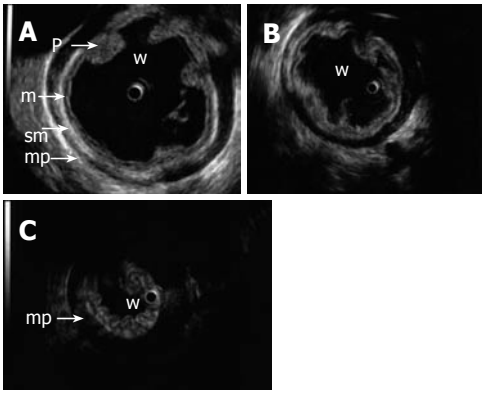


Figure 4 Contraction sequence (**A**, **B**, **C**) demonstrated with a 15 MHz miniprobe in a water-filled (w) stomach. Gastric fold (p), mucosa (m), submucosa (sm), proper muscle (mp).

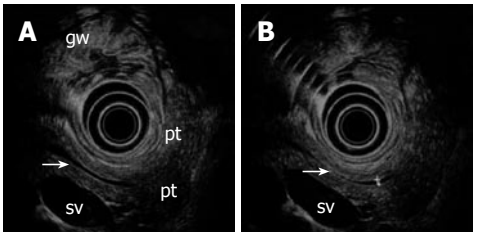


Figure 5 A normal pancreas imaged from the stomach with a 6 MHz echoendoscope. The compressed posterior gastric wall is closely related to the surface of the pancreas. The pancreatic duct is seen (arrow) with minor (**A**) and slightly increased transducer pressure (**B**). The non-compressed gastric wall with folds (gw) is seen on the opposite side of the transducer. Pancreatic tail (pt), splenic vein (sv).

the mucosa accounts for the first, echo-rich layer. The rest of mucosa appears as a second, echo-poor layer. The third, echo-rich layer usually includes the interface between the lamina propria and muscularis mucosae, the submucosa, and the interface at the border of the muscularis propria. In a five-layer structure, the rest of muscularis propria is imaged as an echo-poor fourth layer. The fifth, echogenic layer represents the outer wall delineation and is an interface echo at the serosa or adventitia level.

Sometimes, the use of high-frequency ultrasound produces an interface echo between the different aspects of the muscularis propria, probably reflecting the connective tissue embedding the myenteric plexus. A thick muscularis mucosa may also be identified as an echo-poor layer dividing the third, echogenic layer. Hence, the ultrasonographic wall structure may consist of seven or even nine different layers[6,8].

CLINICAL APPLICATIONS OF ENDOSONOGRAPHY IN BIOMECHANICS AND MOTILITY

Esophagus is often associated with motility disorders which can be studied with different techniques. Endosonography has been regarded as a suitable tool for examination of esophagus because this organ is a straight tube with a small diameter. McCray *et al*[9] studied the high pressure zone between the esophagus and stomach (lower esophageal sphincter and extrinsic crural diaphragm) in normal subjects with a dual high resolution endosonography-manometry catheter. This catheter recorded simultaneously manometric pressures and corresponding ultrasound images while being pulled through the distal esophagus. They found that simultaneous high-resolution endoluminal sonography and pressure manometry separate the two components of the high-pressure zone in the distal esophagus.

Endoscopic ultrasound findings have also suggested that an association exists between sustained longitudinal layer contraction in the esophageal body and chest pain of unknown origin[10]. Furthermore, Nicosia *et al*[11] combined manometry and ultrasound and used conservation of mass principles to quantify local axial shortening of the esophagus. A relationship between contractions in the circular and longitudinal muscle layers during swallowing was established. They also made 3D (space-time) reconstructions of the inner wall of the circular muscle layer for representative swallows. Takeda *et al*[12] used combined ultrasound and manometry to determine circumferential stress-strain properties in the esophagus. In a study by Taniguchi *et al*[13] the sheep esophagus was investigated with M-mode echoesophagram recording and manometry with the goal to measure simultaneously changes in the thickness of the individual esophageal wall layers and the corresponding changes in intraluminal pressure. The maximum manometric pressure, the esophageal wall layer thickness, and the duration of contraction were measured. Swallowing events up to one hour were recorded and in one trial both dry and wet swallows were compared. All swallowing events produced simultaneous increase in intraluminal pressure and esophageal wall thickness. The thickness of the inner (circular) muscle layer increased in dry as well as wet swallows.

The combination of manometry with a 20 MHz ultrasound transducer has also been used to correlate wall thickness, luminal diameter and contractive activity in healthy volunteers[14]. The increase in muscle width by ultrasonography was found to correlate with the increase in luminal pressure by manometry. Recently, Hoff *et al*[15] described a multimodal device inserted into esophagus combining bag distension, manometry, high frequency intraluminal ultrasound, laser Doppler flowmetry and symptom registration. Preliminary experience indicates that these modalities can be used in combination without significant technical influence on each other. Thus, this method can provide a basis for further studies, probably within both the upper and lower GI tract.

Achalasia

Achalasia is a disorder in esophageal motility and is defined by its typical pattern at manometry. The diagnosis can be supported by findings at barium radiography that may show delayed passage through the lower esophageal sphincter and dilatation of the body of the esophagus. At manometry, achalasia is characterized primarily by aperistalsis of the esophageal body and incomplete relaxation of the lower esophageal sphincter. In autopsy

studies of patients with achalasia, the thickness of the lower esophageal sphincter is increased. Using high frequency US probes, it has been shown that the thickening of muscularis propria mainly appear in the inner circular portion (Figure 2). Thickening of the circular layer of the muscularis propria at the cardia may support the diagnosis of evolving achalasia[16-23].

A study of the esophageal wall in patients with newly diagnosed achalasia has also been performed using a sector scanning echoendoscope with 12 MHz transducer frequency. The endosonographic findings were correlated with manometric features and clinical symptoms. In these patients, an increase in total wall thickness and thickness of the muscularis propria was found at the level of the lower esophageal sphincter and the proximal 5 cm of the sphincter[16]. Miller *et al*[21] used a 20 MHz radial ultrasound transducer to examine the width of the total muscularis propria, and the circular and longitudinal smooth muscles at the lower esophageal sphincter in 29 patients with achalasia, comparing them with 19 normal subjects. They found that the mean width of these muscles was increased in patients with achalasia. However, because of overlap in muscle width thicknesses between these groups it was concluded that a thickened muscularis propria cannot be used to differentiate clinically between patients with achalasia and normal controls. Nevertheless, they found that the mean diameter of both the longitudinal and the circular smooth muscle layers at the lower esophageal sphincter were wider in patients with achalasia than in normal subjects. Trowers *et al*[22] used a single crystal transendoscopic probe providing linear images. Six achalasia patients were examined, demonstrating the thickness of the muscularis propria to be increased four-fold, and all patients were abnormal compared to controls.

Scleroderma

Patients suffering from systemic sclerosis have frequently clinical gastrointestinal manifestations, especially excessive deposition of collagen in the esophagus which may cause swallowing problems. Endoluminal US seems capable of defining morphologic as well as physiologic abnormalities of the esophagus in patients with scleroderma of the esophagus. Lieu *et al*[24] examined four patients and found that US images correlated with established pathologic descriptions like smooth muscle atrophy, variable fibrosis, and collagen deposition. Increased echogenicity and thinning of the muscularis propria, consistent with fibrosis and atrophy of the smooth muscle were observed. Miller *et al*[25] used a 20 MHz ultrasound transducer in examining 13 patients suffering from systemic sclerosis and 13 healthy subjects. Also postmortem autopsy study was performed to compare histopathological abnormalities with sonographic findings in the esophagus. In the postmortem ultrasound study of two cases, hyperechoic echoes were seen in the normally hypoechoic muscularis propria. These findings corresponded to fibrosis found in histological examinations. Hyperechoic abnormalities were ultrasonographically seen in the muscularis propria in patients but not in normal controls. A strong positive correlation between esophageal hyperechoic and manometric abnormalities was also found.

Nutcracker esophagus

Patients with nutcracker esophagus (NE) suffer dysphagia and/or chest pain due to strong peristaltic contractions. High-pressure amplitudes are found at manometry. Melzer *et al*[26,27] used an echoendoscope to examine the thickness of the muscularis propria at the gastroesophageal junction and in the lower, middle, and upper esophagus in patients with nutcracker esophagus. The muscularis propria was thickened in one-third of the patients. This thickening, however, did not correspond to the location and magnitude of the manometric abnormality and did not correlate with the clinical presentation. They also reported a patient with NE and esophageal pressures exceeding 800 mmHg in whom endoscopic ultrasonography demonstrated a markedly thickened esophageal muscularis propria. The hypertrophy of the muscularis propria extended from the gastroesophageal junction upwards to the middle esophagus.

Reflux esophagitis

Esophageal dysfunction can be seen in patients with reflux esophagitis. Important pathophysiological factors are impaired esophageal acid clearance and a hypotonic lower esophageal sphincter. The intraluminal esophageal pressure is related to the contraction of the inner circular smooth muscle. Dysfunction of the lower esophageal sphincter area may cause esophageal reflux disease. Contraction of the longitudinal smooth muscle during swallowing causes axial shortening of the esophagus. This muscle may also stiffen the esophagus and thus generate intraluminal pressure during peristalsis. In patients with reflux esophagitis, inflammatory changes are present, affecting all or individual wall layers in the distal part of the esophagus. Periesophageal inflammation may also be present and, depending on the degree of total inflammation, it may affect the esophageal motility and thus also acid clearance. These changes can usually be imaged by ultrasound miniprobes, which thus may provide insights into the pathophysiology of reflux esophagitis.

GASTRODUODENAL MOTILITY

Endosonography of the stomach and duodenum is in essence performed in the same way as in the esophagus. However, endosonography can be technically more challenging in wide and curved organs. Transabdominal ultrasound is applied in studying gastroduodenal motility, also in combination with intraluminal pressure devices. Recently, mechanosensory properties in the human gastric antrum were evaluated using transabdominal ultrasound scanning during volume-controlled antral distension[28,29]. Furthermore, a device which combines endosonographic real - time imaging with intraluminal volume-controlled bag distensions has been developed also for use in large lumina. Thus, detailed images of GI wall layers corresponding to the area of bag pressure can be obtained. Arslan *et al*[30] investigated duodenum with high-frequency ultrasound to study wall layers and motility during allergenic

provocation. A 20 MHz miniature ultrasound probe was inserted through a nasoduodenal tube and ultrasound examination was performed before and after provocation. Clinical symptoms were recorded and the thickness of mucosa, submucosa and muscularis propria was measured directly on ultrasonographic images. Endosonographic changes (i.e. any resolvable wall thickening, contraction or new echogenic layer) were observed in 15 of the 20 patients. Mucosal thickening, changes in the number of wall layers and sustained contractions of duodenum were observed in some patients after provocation. The mucosal thickening could represent mucosal edema in response to provocation. A new echorich layer was observed in two patients, probably caused by an additional interface at the border of a thicker muscularis mucosa due to the allergic reaction.

Rectum, large and small intestine

These organs may be examined in the same way as described for esophagus, stomach and duodenum using dedicated echoendoscopes and rectal probes. However, ES miniprobes, which can easily be inserted through the biopsy channel of standard endoscopes, seem to be a practical method for imaging the colon as well as the rectum. The interpretation of wall layers, organ contractions and other motility aspects is similar to that of the upper GI tract. Endoscopy of the small intestine is now possible in some GI laboratories. New enteroscopes may in the future be integrated with ultrasound transducers making ES/EUS of the small intestine possible. Capsule endoscopy which also is a relatively new method, allows endoscopic imaging of the entire small intestine. If a miniature ultrasound device is integrated in this capsule, EUS of the small intestine may possibly be performed.

NEW TECHNIQUES

Three-dimensional EUS

3D- images are assumed to be easier to understand and communicate than the mental reconstruction of several 2D-images. 3D-techniques are currently under development for most imaging modalities and 3D-EUS may be applied for improved recognition of the GI anatomy and pathological lesions. 3D-EUS images can be obtained using images acquired by echoendoscopes or miniprobes. Mostly, a pullback device has been used to obtain parallel 2D images which are reconstructed to a 3D-image. Acquired ultrasound images can be stored and digitized using frame grabbing but direct digitizing of ultrasound raw data is being increasingly used in new scanners. Advanced software programs containing different rendering or other reconstruction algorithms are used for postprocessing and display of 3D ultrasound data, which can be studied using anyplane slicing, segmentation and volume calculation[31,32].

Elastography

The stiffness of normal and pathological tissue is usually different and may be evaluated by ultrasound which is based on B-mode scanning during compressions (elastography) (Figure 5). Elastography has been applied on the GI tract, kidney, muscle, breast and the heart. The underlying principle is that the deformation of tissue by a mechanical excitation is a function of its mechanical properties. For gastrointestinal purposes, the intraluminal pressure may be used as the excitation force[33,34].

Imaging of ultrasound tissue elasticity has gained considerable interest as a method to differentiate normal from abnormal tissue, particularly malignant from non-malignant disease. However, normal structures also have variable internal sonoelastic properties. When compressed by an ultrasound transducer, the ultrasound image of normal as well as pathological tissue is changed. It has been demonstrated that increased pressure on the GI wall changes the thickness, layers and echogenicity of the wall as imaged by B-mode ultrasonography[7].

Strain rate imaging

Strain rate imaging is a recently introduced technique to measure deformation of biological tissue. The technique is based on tissue velocity imaging, which is an ultrasound technique that provides quantitative information on velocities within a tissue. By color-coded tissue velocity imaging, velocity data from the whole field of view are available simultaneously[35]. This allows extraction of other parameters through spatial and temporal processing of the velocity data. Strain rate and strain are examples of such parameters. The methods have primarily been used in echocardiography, but other uses have also been reported, including measurements of gastric motor function[36].

Further development of specialized EUS instruments can allow the investigators to choose the most appropriate instruments for the separate tasks. Miniature probes, which are less costly and easier to operate, will most likely be available in a majority of endoscopy units within a few years. Different modalities can probably be merged into new multimodal devices making it possible to obtain multiple motility parameters in one procedure. A successful development of such devices can hopefully be achieved on the basis of fruitful collaboration between medicine, technology and soft-ware producers.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, Asbjorn Mohr Drewes, MD, *Series Editor*

New perspectives of studying gastrointestinal muscle function

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Abstract

The motor function of the gastrointestinal tract has primarily been studied using manometry and radiography, though more indirect tests have also been applied. Manometry and radiography do not provide detailed information about the muscle properties as can be assessed from studies of muscle properties in muscle strips in vitro. In recent years a technique based on impedance planimetric measurement of pressure-cross-sectional area relations in a distending bag has proven to provide more detailed information about the muscle function in vivo. This review shows examples of new muscle function analysis such as length-tension diagrams, force-velocity curves and preload-afterload diagrams.

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Key words: Muscle analysis; Length-tension; Force; Velocity

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INTRODUCTION

The gastrointestinal (GI) tract is a continuous channel through the body with oral and anal orifices for intake and output. It consists of a series of organs with the biliary tract as a major side-branch. These organs resemble one another in constitution, the elements being variously arranged in such a way as to produce cylinders, spheroids, or intermediate forms[1]. The wall of the GI tract is a laminated composite structure. The general structure is an outer muscle coat consisting of a longitudinal muscle layer and a circular muscle layer. The collagen-rich submucosa layer and a mucosa layers are found inside the outer muscle coat. A third layer of muscle, the muscularis mucosae, exists throughout almost the entire tract. A network of nerves, the myenteric plexus, is embedded in the loose collagen matrix of the intermuscular plane. This set of nerves is essential in the regulation of the contractions of the adjacent musculature, the two layers of the muscularis propria. The motions of the GI tract must, at all times, keep the contents of the tract in their intended place, i.e. to bring them to the site of absorption. This means not only that they accomplish net antegrade flow but also that they mix the contents and move it across the surface where absorption occurs. The contractile patterns and transit varies greatly from one part of the tract to another. The muscle layers provide the forces that distort the cylindrical conduit in the form of contractions. The connective tissue layers provide a framework, or stroma, that determines the passive physical properties of the gut wall.

As stated above, the gross mechanical function of the GI tract depends on the coordinated muscle function and neuromuscular circuits. In bioengineering terms the mechanical properties of the GI tract has mainly been studied in vitro under standardized isometric and isotonic conditions and length-tension and force-velocity characteristics have been described[2]. The muscle function is altered in a number of organic and functional GI diseases, as well as it can be modified by drugs. Despite the importance of the muscle function, only few methods exist to evaluate the mechanics of GI muscle function in vivo. Manometry has become a standard method for clinical motility testing but it merely provides a proxy of the force developed by the muscles and is in many diseases poorly related to the organ dysfunction and symptoms[3].

Heart muscle function has attracted more attention that GI muscle function in the past and pressure-volume loops and ventricular function curves can be generated from measurements obtained *in vivo*[4,5]. Inspired by the cardiovascular research similar "muscle function" curves can now be obtained in the GI tract from impedance planimetric studies and such analyses provide new insight into the behavior and function of the GI tract. The review provides the background for advanced muscle analysis in vivo and takes the readers through some examples.

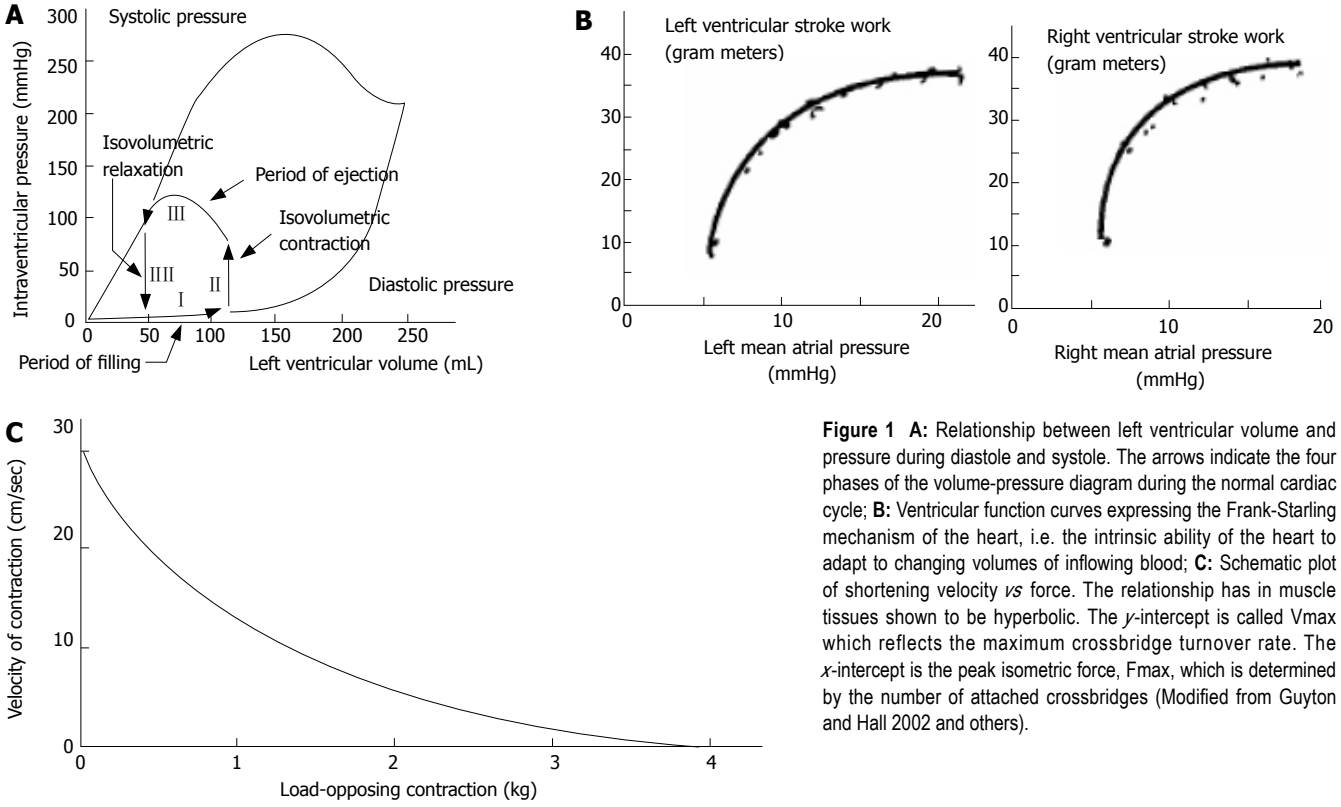


Figure 1 **A:** Relationship between left ventricular volume and pressure during diastole and systole. The arrows indicate the four phases of the volume-pressure diagram during the normal cardiac cycle; **B:** Ventricular function curves expressing the Frank-Starling mechanism of the heart, i.e. the intrinsic ability of the heart to adapt to changing volumes of inflowing blood; **C:** Schematic plot of shortening velocity *vs* force. The relationship has in muscle tissues shown to be hyperbolic. The *y*-intercept is called Vmax which reflects the maximum crossbridge turnover rate. The *x*-intercept is the peak isometric force, Fmax, which is determined by the number of attached crossbridges (Modified from Guyton and Hall 2002 and others).

CURRENT METHODS FOR EVALUATING MUSCLE FUNCTION

The cardiac cycle and cardiac mechanics-function relationship have been studied in much more detail than contractile properties of GI smooth muscle in vivo. Curves are often derived from pressure-volume data where four phases during the cardiac cycle can be identified (Figure 1A), and from stroke work output as function of atrial pressures (Figure 1B). Such data are clinically useful for explaining the pumping mechanics of the left ventricle. One of the best known relationships concerning muscle action is the hyperbolic force-velocity relation[4,6] (Figure 1C). Although this relationship originally was derived for isolated muscle, it has been confirmed for whole striated muscle movements[7]. The curve implies that velocity of muscle contraction is inversely proportional to the load, that a large force cannot be exerted in very rapid movements, that the greatest velocities are attained under conditions of low loading, and that the intermediate values of force and velocity depend on the maximal isometric force.

Most mechanical studies in vitro on GI smooth muscle is isometric length-tension experiments conducted at resting conditions in small organ baths and during stimulation or inhibition with pharmacological substances[2]. Such studies have given valuable information about the regulation of contractions and passive tissue properties.

Only few methods are established for evaluation of GI motor and biomechanical function in vivo. Luminal pressure recordings are the key measurement technique for determining the contractile pattern in many parts of the GI tract. Manometry has been used for more than 50 years to monitor motility in the gastrointestinal tract. These systems often use reliable perfused hydraulic-capillary systems where pressure at a sensor outside the body can be used to accurately measure pressure at a point along a catheter *via* a low flow rate perfused channel. This system has been used extensively to measure force via a pressure measurement in lumens and in liquid filled balloons. Other tests may give a more indirect measure of the motor function. These include transit time studies, pH-metry in the esophagus, radiography, *etc*. The measurement of luminal pressure, however, is not a direct measure of muscle tone and tension. It is a proxy of changes in force generated by the surrounding tissues[8,9].

During recent years, bag distension has become popular as a research tool for GI studies, primarily because mechanical data may be obtained. Furthermore, distension provokes pain and therefore can be used to study the sensory function in patients with functional chest pain[10]. Only few distension studies using inhibitory drugs focused on separating phasic and tonic contractions[11,12]. Recently, tools were developed for studying both the active (phasic and tonic contractions) and passive behavior using impedance planimetric distension before and during administration of inhibitory drugs[9,13,14]. Tension, stress and strain are of principal interest because these parameters give information about the esophageal elastic properties and mechanoreceptors respond more directly to these parameters than to pressure and volume[9]. Furthermore, the muscle function is better evaluated when the forces and tensions can be measured rather than the pressure generated by contractions[1,15]. The most used bag distension technique is the barostat but other techniques such as impedance planimetry and distensions combined

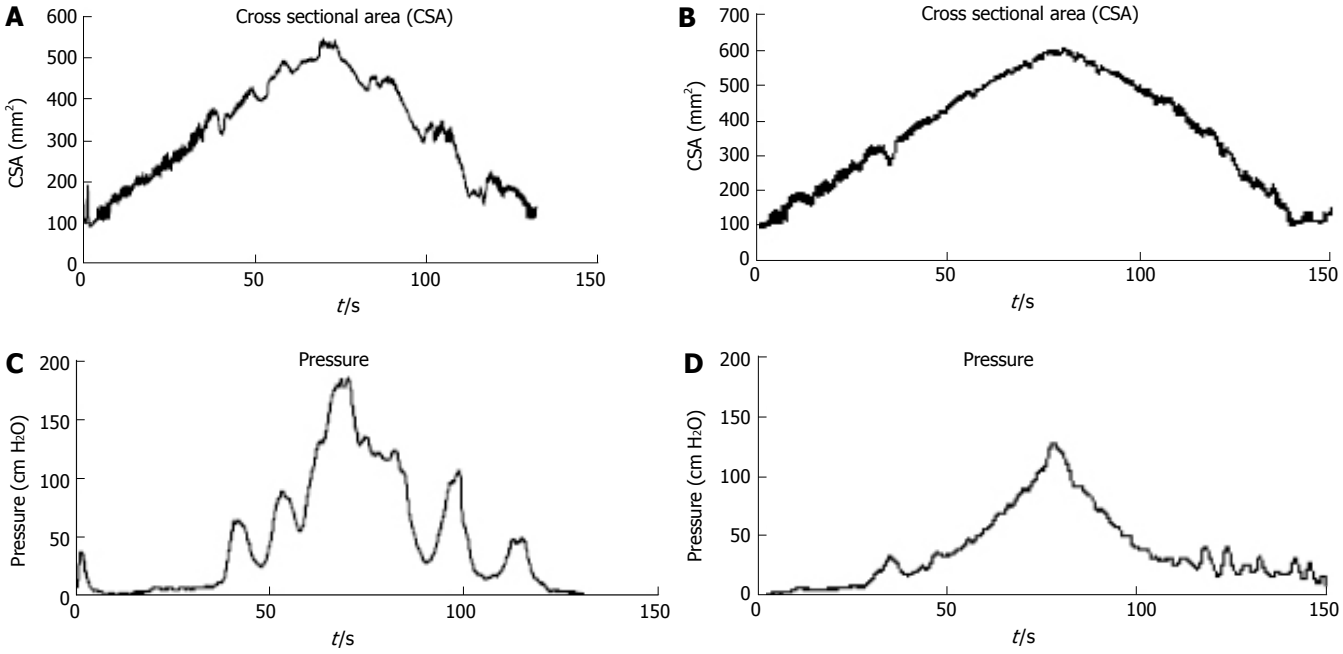


Figure 2 Cross-sectional area (CSA), and pressure as function of time during the distension of esophagus without (**A, C**) and during (**B, D**) the administration of butylscopolamine. Modified from previous publications by Gregersen and coworkers.

with imaging techniques give more detailed geometric information. Rather than analyzing pressure-volume relationships based on the barostat, impedance planimetry enables data on the CSA of the bag during distension to be gathered. CSA shows a better relationship to the diametrical changes in the alimentary lumen than volume[1].

THE IMPEDANCE PLANIMETRIC PRINCIPLE

Impedance planimetry is a technique for performing bag distensions in hollow visceral organs. It has been used to measure CSA together with bag pressure in animal and human studies and the wall tension and strain in the organ can be estimated[1,16]. From Ohm´s law we know that

$$V = IZ$$

where V, I and Z denote the potential difference, the current and the electrical impedance. In the case of a fluid filled bag on a probe with an alternating constant current set up between two excitation electrodes (E), Ohms law will apply for the field between two inner sensing electrodes (S). The impedance depends on the distance (D) between the S electrodes, the conductivity (ς) of the liquid in the bag and the CSA of the bag between the S electrodes. Since

$$Z = \frac{D}{\varsigma CSA}$$

the potential difference can be written as

$$V = \frac{DI}{\varsigma CSA}.$$

If the conductivity of the solution remains constant (the temperature must be constant), the distance between the S electrodes is fixed, and the current source exciting the E electrodes is kept constant, then

$$V = \alpha \frac{1}{CSA},$$

where α is a calibration constant. Thus, the voltage crossing the S electrodes is proportional to the inverse of the CSA. Direct proportionality can be obtained by reciprocation. Data are conditioned and stored on a PC for further analysis.

EXAMPLES OF MUSCLE ANALYSIS

The new analysis consist of *in vivo* (1) phasic and tonic length-tension diagrams; (2) preload-afterload analysis (3) pressure-CSA and tension-radius plots (4) force-velocity and force-power plots.

Basic distention studies

The studies have been done using different step or volume-controlled ramp protocols. An example of CSA and pressure recordings is shown in Figure 2 where a volume-controlled ramp distension was done using an infusion rate of 25 mL/min. The data provide information on the contractile pattern and can be used for computation of elasticity.

Assessment of phasic, tonic and passive length-tension relationship in vivo

Length-tension diagrams can be obtained in vivo in the GI tract using impedance planimetry. Passive curves obtained during administration of butylscopolamine and active curves in terms of both phasic and tonic curves can be generated by analyzing data in accordance with the tracings in Figure 3. For simplicity in the first approach, the circumferential wall tension (T) is calculated according to the law of Laplace for cylindrical structures.

$$T = \Delta P r$$

where T is the circumferential wall tension and r is the bag radius and ΔP is the transmural pressure. The radius is calculated assuming a cylindrical shape of the bag during

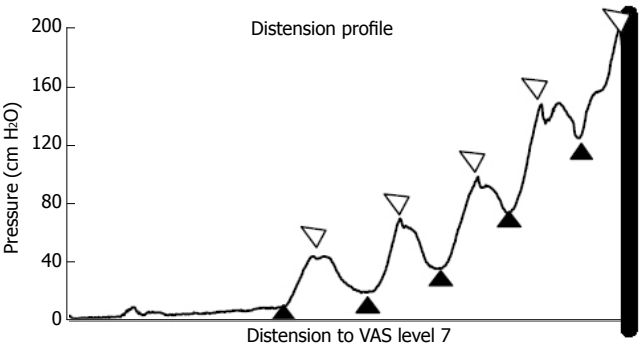


Figure 3 Illustration of a ramp distension curve in the human esophagus. The thick line at the end of the curve is the reverse point. The open symbols above the curve mark the phasic part and the afterload pressure. The closed symbols under the curve mark the tonic part of the distension curve and the preload pressure. The passive pressure is measured from the tonic part of the curve during administration of butylscopolamine. At the symbols pressure and CSA was measured and radius and tension was computed from these values. Modified from previous publications by Gregersen and coworkers.

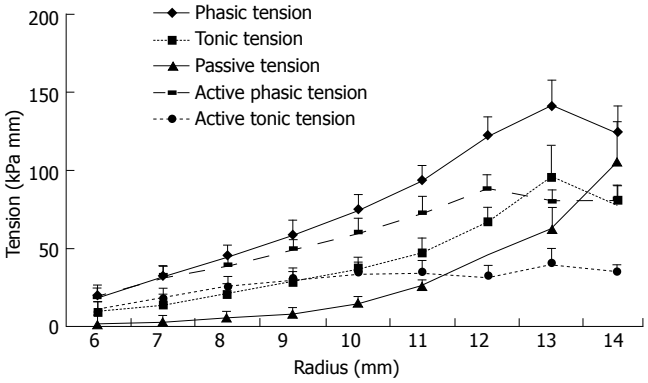


Figure 4 The active and passive tension-radius curves for a subject where the maximum active tension is reached before the moderate pain level (top). The bottom graph shows the averaged data from all volunteers. Mean and SEM values are shown. Modified from previous publications by Gregersen and coworkers.

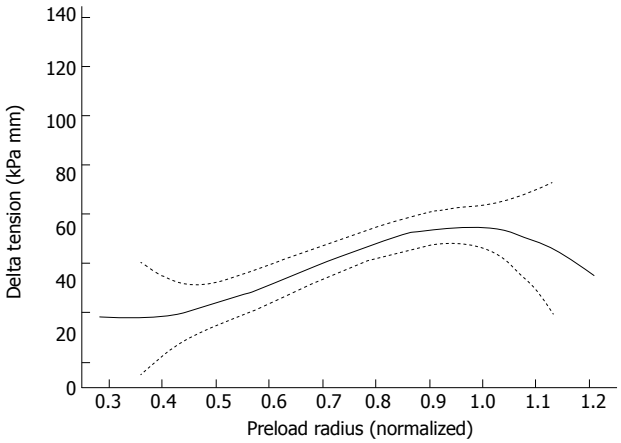


Figure 5 Representation of the change in muscle tension during distension-induced contractions as function of the radius immediately before the contraction (preload-afterload properties) of the esophageal muscles. The solid line is the polynomial fit and the dotted lines represent 95% confidence intervals. Modified from previous publications by Gregersen and coworkers.

distension and by knowing the CSA. The circumferential stretch ratio can be computed as the fractional change in radius (r/r_0) where r is the radius at any distension state and r_0 is the reference radius.

The distentions induce contractile activity. For each contraction, tonic tension (tension computed from pressure and CSA between phasic contractions) and phasic tension (tension during maximum contraction) can be identified from the tension-time curve. Since both active and passive tissue properties contribute to the total tension developed in the small intestine during distension, both the tonic and the phasic tensions must be separated into active and passive components. The passive tension can be derived from the distensions during butylscopolamine infusion and the total tension. Thus, the active tensions can be computed as:

$T_{tonic\text{-}total} = T_{tonic\text{-}active} + T_{passive}$ and $T_{phasic\text{-}total} = T_{phasic\text{-}active} + T_{passive}$

where $T_{\text{tonic-total}}$ $T_{\text{tonic-active}}$, $T_{\text{phasic-total}}$ $T_{\text{phasic-active}}$ and T_{passive} are the total tonic tension, the active tonic tension, the total phasic tension, the active phasic tension, and passive tension, respectively.

The graph in Figure 4 shows averaged data from the normal human esophagus. The passive tension increased exponentially as function of the radius, whereas the tonic and combined phasic-tonic tension increased until a maximum at a radius of 13 mm. This corresponded with the pain threshold. The active tension decreased at higher radii. The major finding was that both active curves exhibited a maximum tension. The active tensions were most important at low distending loads whereas the passive tension was predominant at higher loads. Thus, the esophagus can contribute its largest active force to transport the bolus within the physiological range. The maximum active tension appears close to the pain threshold and before overstretching the esophageal wall. Thus, the esophagus has its physiological function in the ascending leg of the length-tension curve. Exceeding this level it becomes inefficient, like the heart that also functions at the ascending leg of the curve. The maximum active tension is presumably reached at a level of optimum overlap between the sliding filaments in the intestinal muscle cells. When the esophageal wall was stretched to a degree where pain appeared, the passive tension predominated. The passive curves were exponential-like in accordance with findings in other soft tissues. The exponential behavior protects the tissue against overdistension and damage at high luminal pressure loads, while distending easily and facilitating flow in the physiological pressure range.

Preload-afterload diagrams

A way to express the Frank-Starling mechanism in the heart is preload-afterload curve (Figure 1B). In Figure 5 this concept was borrowed from cardiac physiology and it is shown that GI muscle behaves in a similar fashion. The change in tension during individual distension-induced contractions (afterload) was analyzed as function of the radius immediately before the contractions (preload radius). In a similar way the change in radius during distension-induced contractions was plotted as function of the preload radius. These diagrams correspond to heart function curves in terms of the ventricular stroke work as function

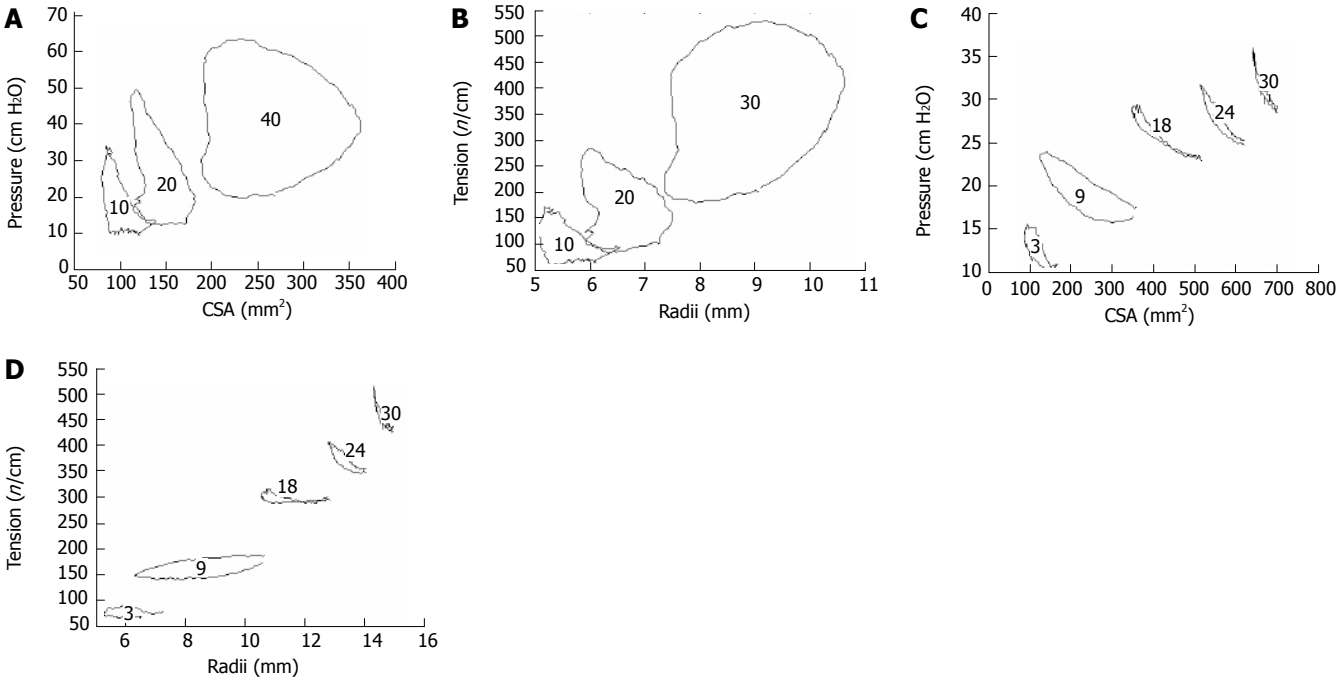


Figure 6 Pressure-CSA and tension-radius relations during esophagus contractions (**A, B**) and during duodenum contractions (**C, D**). The numbers in the figures indicate the volume in ml.

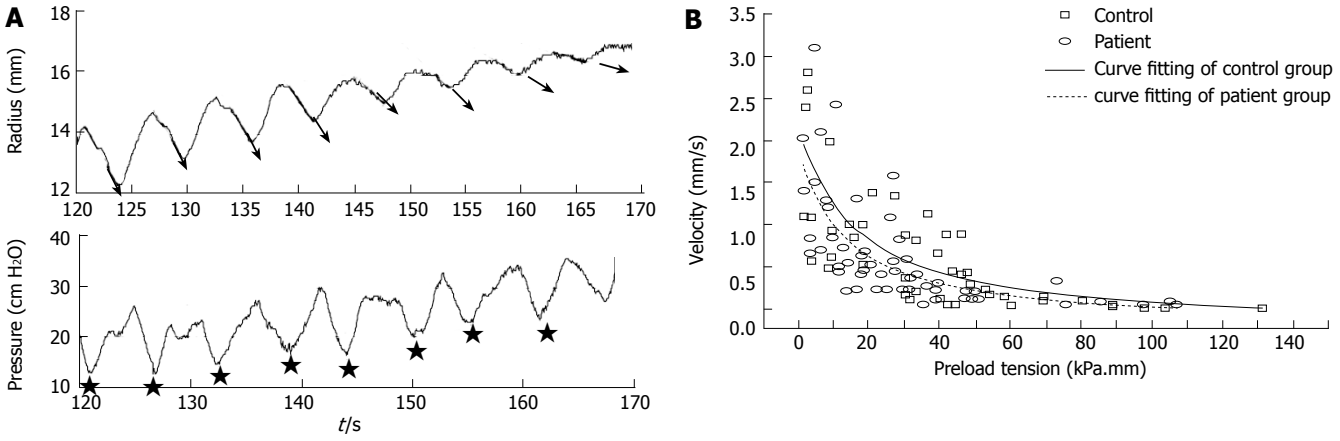


Figure 7 **A:** Arrows indicate that the slope (velocity decreases at high loads. The stars indicate the preload tension; **B:** Force-velocity curves represented as circumferential preload tension-radius shortening velocity and preload tension-circumferential preload tension*CSA rate in SS patients (symbol O and dashed line) and controls (and solid line). The tension-velocity data were curve fitted using Hill's equation. Modified from previous publications by Gregersen and coworkers.

of the mean atrial pressure. Such curves demonstrate the Frank-Starling mechanism of the heart[4,5]. The change in tension during bag-distension-induced contractions increased at normalized radii (radius divided by the radius at the pump reverse point) up to 0.95 (corresponding to radii of 5-12 mm) (Figure 5). This may be called the ascending leg of the curve. At higher radii the change in tension decreased again (the descending leg).

The preload is considered to be the initial muscle length (radius) preceding the contraction during the distension, whereas the afterload is evaluated as the active tension during the contraction. In cardiac physiology the preload is usually considered to be the end-diastolic pressure or radius and the afterload is considered to be the arterial pressure during the systole. The explanation of the Frank-Starling mechanism is that when an extra amount of blood flows into the ventricles, the cardiac muscle itself is stretched to greater length. This causes the muscle to contract with increased force because the actin and myosin filaments are brought to a more nearly optimal degree of interdigitation for force generation. In cardiac physiology the importance of the concept of preload and afterload is that in many abnormal functional states of the heart and circulation, the pressure during filling of the ventricle or the arterial pressure against which the ventricle must contract, or both, are severely altered from the normal. Transferring this concept to GI physiology, the development may have interest for evaluation of normal GI physiology, aging and in the pathophysiology of gastro-esophageal reflux disease, systemic sclerosis, obstruction, achalasia, functional chest

pain, spasms and other esophageal disorders.

P-CSA and tension-radius plots

These curves correspond to the well known pressure-volume curves obtained in the heart (as shown in Figure 1A. The data below was generated from analyzing pressure and CSA data during contractile events at various levels of bag distension in the esophagus and duodenum (Figure 6). It is clearly shown that loops consisting of different "phases" can be determined. However, definition of contraction and relaxation phases seems not as clear cut as in the heart. It is evident that duodenum and esophagus muscle behaves in a very different way. Especially the esophagus curves show a large degree of hysteresis. The area enclosed by the tracing is a measure of the work done during the contraction.

Force-velocity analysis

Figure 1C shows a schematic of the well known force-velocity relationship for muscle tissue and Figure 7 shows data obtained in the GI tract. The tension and shortening velocity were quantified from the contraction waves in the tension-time and radius-time curves simultaneously. For each contraction the preload tension was defined as the tension preceding the contraction. The shortening velocity was defined as the radius shortening rate which is the slope of the radius-time curve for each contraction (arrows in Figure 7A). Since it turned out that the tension-shortening velocity relationship was a hyperbola similar to that described for skeletal muscle, Hill's equation can be used to characterize this relationship[5].

$(T + a)(V + b) = (T\max + a)b$

where T represents tension, V the velocity of shortening, Tmax the maximum tension at which the muscle is first unable to move, a and b are constants with dimensions of force and velocity, respectively. Figure 7A shows the bag radius (derived from the CSA) and the pressure during ramp distension of the duodenum. The slope of the contraction curve (arrows representing the contraction velocity) decrease as a function of the degree of distension. Force-velocity curves expressed as circumferential preload tension-radius shortening velocity diagrams are shown in Figure 7B for the duodenum in controls and systemic sclerosis patients. The velocity of shortening decreased with increasing preload tension. *T*max and the muscle power were lowest in the patients and association was found between the duration of the disease and the maximum stretch ratio. Decreased muscle function may explain the GI symptoms reported in this patient group and should be considered as an important factor in the diagnosis and treatment.

In conclusion, current understanding of the "mechanical" physiology of the GI tract is based on measurement techniques such as manometry. The development of new analysis of bag distention data will contribute significantly to the understanding of digestion in health and disease in the future. The impact of the proposed analysis is to better understand the muscle properties and how they relate to mechanism involved in symptom generation in organic and functional diseases in visceral hollow organs. Examples of frequent diseases of great interest are GERD, non-cardiac chest pain and functional dyspepsia. The perspective is that future treatment of GI disorders with affection of neuromuscular pathways can be mechanism- and evidence based with respect to such function curves.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, *Series Editor*

Three-dimensional surface model analysis in the gastrointestinal tract

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Abstract

The biomechanical changes during functional loading and unloading of the human gastrointestinal (GI) tract are not fully understood. GI function is usually studied by introducing probes in the GI lumen. Computer modeling offers a promising alternative approach in this regard, with the additional ability to predict regional stresses and strains in inaccessible locations. The tension and stress distributions in the GI tract are related to distensibility (tension-strain relationship) and smooth muscle tone. More knowledge on the tension and stress on the GI tract are needed to improve diagnosis of patients with gastrointestinal disorders. A modeling framework that can be used to integrate the physiological, anatomical and medical knowledge of the GI system has recently been developed. The 3-D anatomical model was constructed from digital images using ultrasonography, computer tomography (CT) or magnetic resonance imaging (MRI). Different mathematical algorithms were developed for surface analysis based on thin-walled structure and the finite element method was applied for the mucosa-folded three layered esophageal model analysis. The tools may be useful for studying the geometry and biomechanical properties of these organs in health and disease. These studies will serve to test the structure-function hypothesis of geometrically complex organs.

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Key words: GI tract; Surface; Tension; Stress; Three-dimensional

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INTRODUCTION

Biomechanical changes due to functional loading and unloading of the human gastrointestinal (GI) tract are not fully understood. The GI function is usually studied by introducing probes in the GI lumen, e.g. for pressure recordings. Computer modeling based on medical imaging offers a promising alternative approach in this regard, with the additional ability to predict regional stresses and strains in inaccessible locations. Thus, the three-dimensional (3-D) geometry of visceral organs is of considerable interesting since the morphology and geometry are changed in various diseases[1,2]. The tension and stress distributions in the GI tract are related to distensibility (tension-strain relationship) and smooth muscle tone. The most widely used method for biomechanical stimulation of the gastrointestinal tract is bag distension[3,4]. Distension data are traditionally based on pressure-volume measurements[5-9]. Several studies suggest that the mechanoreceptors located in the gastrointestinal wall are tension receptors[10,11]. Distension technology has been refined by the introduction of the impedance planimetry technique and the endoscopic ultrasound probe[12-23]. These are now well-established methods for assessing circumferential strain, tension, stress and stiffness (slope of stress-strain relation). Previous GI studies have provided data on wall tension and stress in visceral organs. These studies, however, were based on two-dimensional models; i.e., the circumferential tension and stress have been determined at one specified position along the long axis of the organ. However, the tension distribution is related to the geometry (radii of curvature) and the distension pressure according to Laplace's law. Complex geometries will result in heterogeneous tension distributions. Therefore, three-dimensional geometry based on medical images is necessary for computing the tension and stress in the GI tract at various physiological and pathophysiological states.

Recently, 3-D surface model of the gall bladder, stomach, rectum and sigmoid colon have been developed by simultaneous cross-sectional imaging using ultrasonography, computer tomography (CT) or magnetic resonance

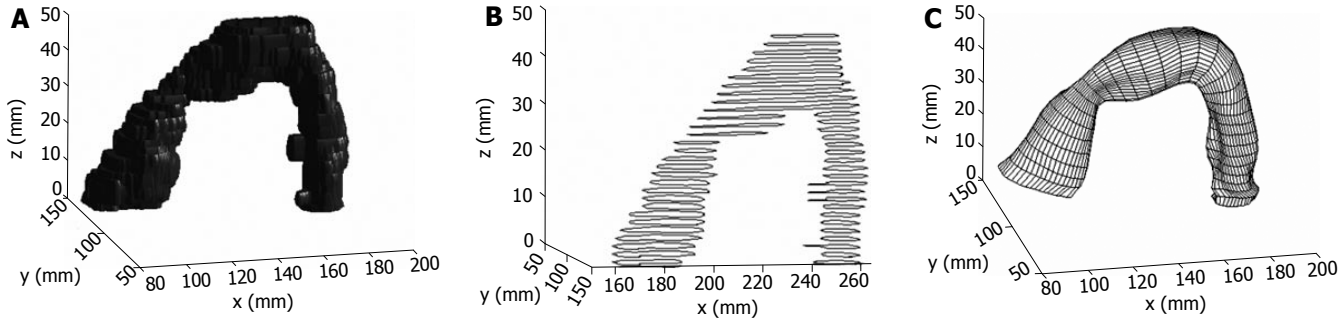


Figure 1 A 3-D sigmoid colon solid model (**A**), the surface model based on the cross sectional images (**B**)and the 3-D surface model with slices along the centre axis (**C**).

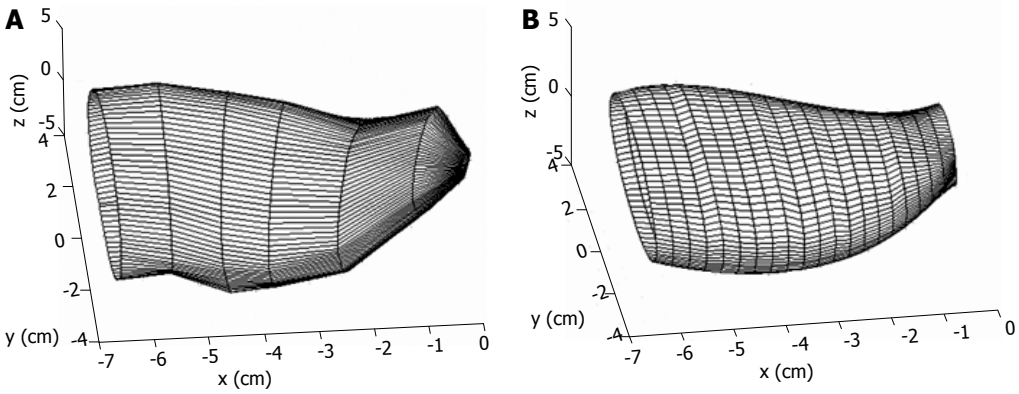


Figure 2 A 3-D reconstructed stomach antrum wall surface before (**A**) and after (**B**) surface smoothing by using the Fourier transform method.

imaging (MRI)) and pressure measurement during distension[24-27]. Using this method it is possible to model the 3-D geometry and mechanical properties of the gastrointestinal tract, including the spatial distribution of the 3-D principal curvatures, radii of curvature, wall thickness, tension and stress.

Examples of 3-D surface models of the GI organs, including stomach, rectum, sigmoid colon and gall bladder and the mucosal folded three layered esophageal finite element model are presented. Two different mathematic algorithms used for surface analysis were introduced. The human stomach[24] and gall bladder[25] models were generated based on the cross sectional images and the surfaces were analyzed theoretically by using a Fourier transform method. The human rectum[26], human sigmoid colon and rat stomach[27] were established from re-sliced images. The surfaces were analyzed locally by using a numerical method.

GEOMETRIC MODELS

Image acquisition and model reconstruction

The anatomical data used for the construction of the initial GI 3D models were obtained from ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI). The image processing method was used to identify the inner and outer wall boundaries and then a data cloud of a 3-D model was obtained. A 3-D sigmoid colon model built from cross sectional images is illustrated in Figure 1.

For GI organs such as the stomach[24], rectum[26] or sigmoid colon (Figure 1), the centre axis are curved. The distended organs are deformed along the curved axis. Alignment of data points along this curved centre axis was necessary to describe the GI model deformation. Based on a 3-D solid model generated from the cross sectional images, it can be re-sliced along the curved centre axis and the surface model based on the re-sliced images can be obtained. Figure 1 show a sigmoid colon solid model and a surface model generated from the re-sliced images.

Surface smoothing

The reconstructed surfaces have some irregularities due to the discretization of the images. The irregularities were removed using two different smoothing methods in our studies, one method was based on the Fourier transform algorithm and another one is a modified non-shrinking Gaussian smoothing method[28]. The two smoothing algorithms used in the surface analysis are illustrated in Appendix A and B. By using the algorithm described in appendix A, a complex surface can thus be described and smoothed by the surface function of Eq. A3. A smoothed stomach antrum model is illustrated in Figure 2. Figure 3 shows a sigmoid colon model that was smoothed based on the Gaussian smoothing method. With the continuous and smoothed surface, thus the surface can be approximated locally by a biquadric surface patch. The parametric surface used in our studies is a tensor product B-spline surface which gives the necessary continuity properties and with good surface approximation[29].

GEOMETRIC ANALYSIS

Curvature calculation

With the surface function Eq. (A3) or Eq.(B1) at the local co-ordinate system, the principle curvatures of

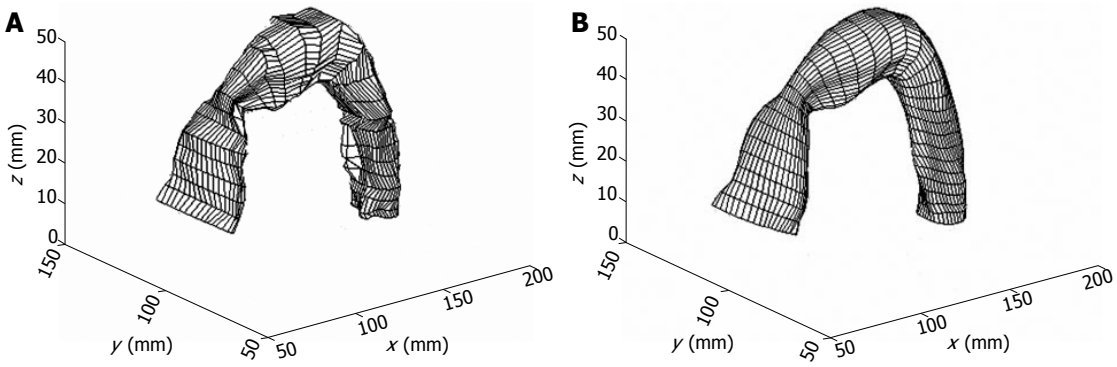


Figure 3 A 3-D sigmoid colon segment surface before (**A**) and after (**B**) surface smoothing by using the non-shrinking Gaussian method.

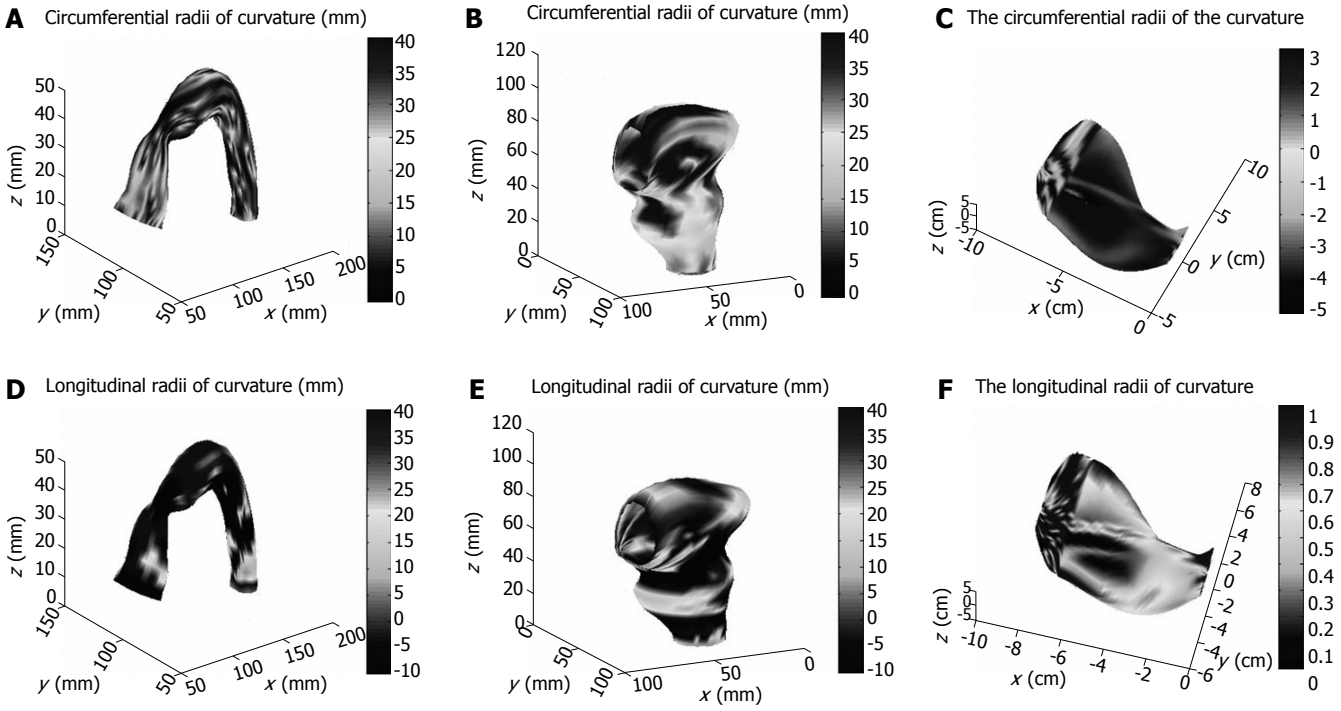


Figure 4 Circumferential (**A, B, C**) and longitudinal (**D, E, F**) radii of curvatures distributions in sigmoid colon (**A, D**), rectum (**B, E**) and human stomach (**C, F**).

the surface were generated from the coefficient of the first fundamental form (E,F and G) and the second fundamental form (L, M and N) of the surface function. The principal curvatures κ_1 and κ_2 can be combined from the *Gaussian* curvature (K_G) and the *Mean* curvature (K_M):

$$K_G = \kappa_1\kappa_2 = \frac{LN - M^2}{G - F^2} \quad (1)$$

$$K_M = \frac{1}{2}(\kappa_1 + \kappa_2) = \frac{1}{2}\frac{NE - 2MF + LG}{EG - F^2} \quad (2)$$

K_G is a particularly useful curvature parameter that indicates an elliptical surface ($K_G > 0$), a parabolic surface ($K_G = 0$) or a hyperbolic surface ($K_G < 0$).

Circumferential and longitudinal curvatures distribution on human sigmoid colon, human rectum and human stomach models are illustrated in Figure 4.

Tension and stress calculation

If we assume that the GI organs are thin-walled and its material properties are isotropic, the surface tension at a surface point is related to the transmural pressure by Laplace's equation as follows:

$$P = T[\kappa_1 + \kappa_2] \quad (3)$$

where κ_1 and κ_2 are the principal surface curvatures at a surface point. This equation is valid as long as the membrane is so thin that bending rigidity can be neglected and the curvature changes are not sudden. Accordingly, only the spatial points with positive Gaussian curvature were used for tension (and stress) calculations in this study. Stress is defined as the force acting on a unit area. The stress distribution on the surface can be calculated from wall tension and normal wall thickness as:

$$\sigma = T/h \quad (4)$$

T is the surface tension and h is the normal wall thickness. An example of tension and stress distribution on the rectum model are illustrated in Figure 5.

FINITE ELEMENT ANALYSIS FOR MUCOSAL FOLDED LAYRED ESOPHAGUS

The major shortcoming of the above surface analysis is the thin wall assumption. Thus only surfaces with positive principal curvatures in both directions could use Laplace's

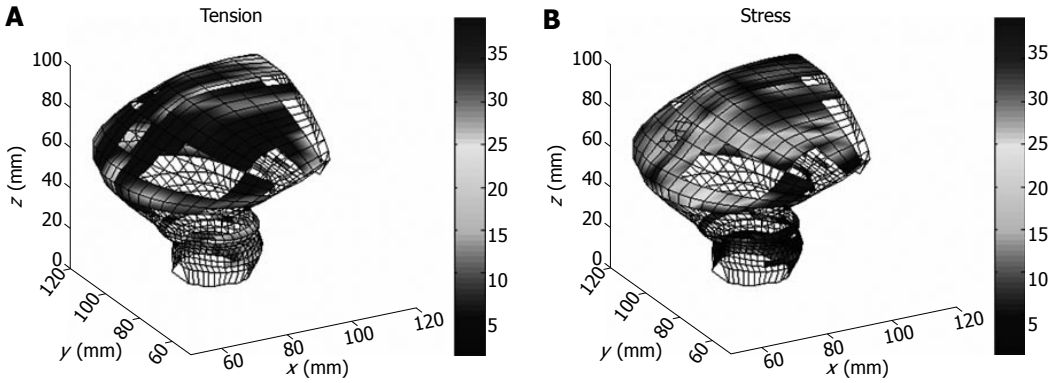


Figure 5 Tension (**A**) and stress (**B**) distribution on a distended rectum model.

equation for tension and stress calculation (Figure 5). To overcome this limitation, numerical methods such as finite element analysis must be adopted. The finite element method has been used for modeling GI tract bioelectric activity[30,31] and the layered esophagus distension simulation[32,33]. Figure 6 is a mucosal folded three layered esophageal finite element model. It is indicated that the stress distribution throughout the esophageal wall was not uniform and the stress was discontinuous between the muscle and mucosal-submucosal layer (Figure 6). Thus the Laplace's law is no longer valid in such layered and thick wall models.

PERSPECTIVES

Mechanosensitive receptors are important for GI tract function and sensation. The intraganglionic laminar nerve ending (IGLE) is believed to be a mechanosensitive receptor and is located within the smooth muscle-lined regions of the GI tract[34,35]. It is generally believed that they are tension receptors. The neurons respond in an intensity-dependent manner to the gastric distension[35,36]. Hence, determination of the stress or tension locally is important for understanding the mechanical environment at receptor sites. Methods based on pressure-volume measurements obtained during bag distension (such as the barostat) do not directly measure variables used in plane stress and strain analysis[5,8,12,22]. Because the bag can elongate, cross-sectional area cannot be derived from bag volume. Moreover, the main assumption for the computation of tension using the barostat and Laplace's law is that the geometry is spherical, although it seems obvious from medical imaging modalities such as ultrasound and magnetic resonance (MR)-scanning of the complex GI organs such as stomach or rectum that the assumption is not valid. Impedance planimetry measures luminal pressure and cross-sectional area in a short segment of hollow viscera during bag distension[14-18,22,37]. Based on these measures the circumferential strain, tension (applying Laplace law) and stiffness (slope of tension-strain relation) of a specific cross-sectional segment can be computed. This approach has limitations in irregular organs like the stomach, rectum or sigmoid colon because the calculated tension is based on the assumption of circularity, is only valid for thin walled structures, and only accounts for circumferential tension.

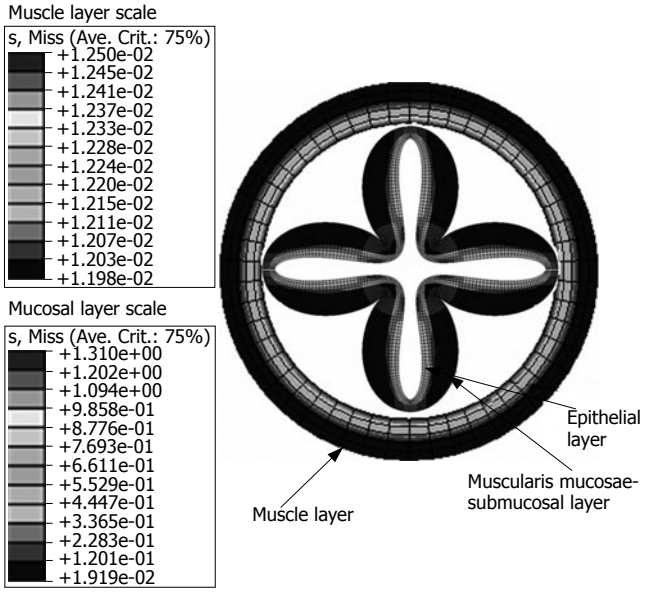


Figure 6 Mises stress (left) distribution in the mucosal folded three-layered esophagus at the bonded state.

In our 3D surface analysis, we generated the organ geometry based on 3-D ultrasound or MR images measurements. It is clear that GI organs such as the stomach, rectum, sigmoid colon and gall bladder have heterogeneous curvature distributions. Consequently, the geometry cannot be assumed spherical or cylindrical and that tension estimations based on Laplace's law are dubious because the geometry of the surface is complex. Two different methods were used for building 3D model and the surface approximation. By using the Fourier transform method the whole surface was reconstructed and smoothed simultaneously, i.e. the surface can be described theoretically and then the curvatures can be computed with good accuracy. However, it is difficult to describe all geometry characters in such complex GI organs by using only one function. Furthermore, the Fourier transform method can only be used on the surface generated from the horizontal cross sectional images. The analysis employed in these surface models produces large errors at both polar ends, especially to models with a curved center axis, such as the stomach, rectum or sigmoid colon. Since 3D solid model can be re-sliced in any direction, a new method for re-generating the 3-D surface

from the solid model was developed. By using the new method, all geometric characters can thus be kept in the surface model.

The major limitation of the surface analysis is the thin wall assumption. Thus only surfaces with positive principal curvatures in both directions could use Laplace's equation for tension and stress calculation. The finite element analysis or the finite difference method should be developed for further GI modeling analysis in the future. 3-D modeling based on cross-sectional imaging (CT, MRI and ultrasonography) has been used to characterize biomechanics and geometry in for instance the stomach[24,27], the rectum[26] cardiovascular system[38], and in computer-aided orthopedic surgery. The present analytical tool can be used to analyze complex structures for understanding biomechanical properties and visceral perception in other hollow visceral organs. The geometry and sensory aspects of diseases in the sigmoid colon (diverticular disease, irritable bowel syndrome, *etc.*), small bowel (motility disorders), stomach (motility disorders, non-ulcer dyspepsia, *etc.*) and esophagus (esophagitis, gastro-esophageal reflux disease, non-cardiac chest pain, *etc.*) can be obtained by applying a modified version of this method.

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APPENDIX A

Surface smoothing based on the Fourier transform method

Arbitrary point in a given cross section of the surface can be expressed in a polar co-ordinate system as (r, θ, z), where r, θ and z are the polar radius, angle and section height with respect to the origin. Each point can be fitted using a Fourier series as

Math1

$$r(\theta) = a_0 + \sum_{n=1}^{n=d} a_n \cos(n\theta) + \sum_{n=1}^{n=d} b_n \sin(n\theta) \qquad \text{(A1)}$$

where d denotes the degree of the Fourier series.

To approximate the Fourier coefficient as a function of the section height z, orthogonal polynomial were used as

$$a_i(z) = c_{0i} + c_{0i} z + c_{2i} z^2 + \cdots + c_{pi} z^p \qquad \text{(A2)}$$

where p is the degree of the polynomial and i is the index of the Fourier coefficient. The (r, θ, z) coordinates were described using vector rotation as

$$X(\theta, z) = (r(\theta, z)\cos(\theta), r(\theta, z)\sin(\theta), z) \qquad \text{(A3)}$$

where

Math2

$$r(\theta, z) = \begin{bmatrix} 1 \\ \cos\theta \\ .... \\ \sin d\theta \end{bmatrix}^T \begin{bmatrix} c_{00} & \cdots & c_{p0} \\ c_{01} & \cdots & c_{p1} \\ .... & ... & .... \\ c_{0(2d+1)} & \cdots & c_{p(2d+1)} \end{bmatrix} \begin{bmatrix} 1 \\ z \\ .... \\ z^p \end{bmatrix} \qquad \text{(A4)}$$

Hence, all the information of the surface is contained in the coefficient matrix, i.e., the surface was now transformed from a three-dimensional Cartesian coordinates (x, y, z) to a two-dimensional local tangent plane coordinates system (θ, z), where the θ is the tangent direction corresponding to the circumferential muscle direction and z to the longitudinal muscle direction, respectively.

APPENDIX B

Surface smoothing based on modified non-shrinking Gaussian method

This smoothing algorithm consists of two consecutive Gaussian smoothing steps with a positive scale factor λ applied to all the vertices of the shape, a second Gaussian smoothing step applied to all the vertices but with a negative scale factor μ, greater in magnitude than the first scale factor ($0 < \lambda < -\mu$). To produce a significant smoothing effect, these two steps must be repeated, alternating the positive and negative scale factors, a number of times. The relation between the position of the vertices of the surface before and after N iteration can be expressed as

$$X^N = ((I - \mu K)(I - \lambda K))^N X \qquad \text{(B1)}$$

where N was the number of iterations, λ and μ are two scale factors, I is the $n_V \times n_V$ identity matrix, $K = I - W$, W is the weight matrix and n_V is the number of the neighborhood of a vertex. In fact this method produces a low pass filter effect. The two scale factors determine the pass band and the stop band.

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REVIEW

Immune therapy including dendritic cell based therapy in chronic hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) infection is a global public health problem. Of the approximately 2 billion people who have been infected worldwide, more than 400 million are chronic carriers of HBV. Considerable numbers of chronic HBV carriers suffer from progressive liver diseases. In addition, all HBV carriers are permanent source of this virus. There is no curative therapy for chronic HBV carriers. Antiviral drugs are recommended for about 10% patients, however, these drugs are costly, have limited efficacy, and possess considerable side effects.

Recent studies have shown that immune responses of the host to the HBV are critically involved at every stage of chronic HBV infection: (1) These influence acquisition of chronic HBV carrier state, (2) They are important in the context of liver damages, (3) Recovery from chronic HBV-related liver diseases is dependent on nature and extent of HBV-specific immune responses. However, induction of adequate levels of HBV-specific immune responses in chronic HBV carriers is difficult. During the last one decade, hepatitis B vaccine has been administered to chronic HBV carriers as a therapeutic approach (vaccine therapy). The present regimen of vaccine therapy is safe and cheap, but not so effective. A dendritic cell-based therapeutic vaccine has recently been developed for treating chronic HBV infection. In this review, we will discuss about the concept, scientific logics, strategies and techniques of development of HBV-specific immune therapies including vaccine therapy and dendritic cell-based vaccine therapy for treating chronic HBV infection.

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Key words: Hepatitis B virus; Dendritic cells; Immune therapy; Vaccine therapy

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INTRODUCTION

Hepatitis B Virus (HBV) is a hepatotropic, noncytopathic, DNA virus which can cause acute as well as chronic infection. Chronic HBV infection remains a significant public health problem world-wide. It is estimated that there are at least 400 million HBV carriers in the world and that up to one million die annually due to HBV-associated liver diseases. Although the majority of the chronic HBV carriers may remain in an inactive phase associated with low viral replication and histological remission, a significant proportion will develop chronic hepatitis B (CHB), which is characterized by the presence of high viral replication, and chronic necroinflammation of the liver. Patients with CHB develop serious complications like liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Indeed, all chronic HBV carriers are at higher risk for HCC when compared with general population; the risk becomes much higher when LC develops. In addition, all chronic HBV carriers (inactive HBV carrier, patients with CHB [both treated and untreated], patients with LC and HCC) are permanent and living sources of HBV. Presence of million of chronic HBV carrier is practically responsible for the incidence of acute and fulminant hepatitis due to HBV[1].

No specific treatment is provided to 90% of chronic HBV carrier ('do nothing strategy'). Antiviral drugs are recommended only for patients with high HBV replication and necroinflammatory liver diseases. These drugs include type-1 interferons (IFN) and nucleosides analogues such as lamivudine and adefovir. It is difficult to assess the real therapeutic impact of antiviral drugs in chronic HBV carriers. On one hand, these drugs are recommended in some selected patients with CHB. On the other hand, the response varies considerably depending on virological and immunological statuses of the patients. Finally, many terms such as complete responder, biological responder, virological responder, transient responder, and sustained responder are used to assess responses to antiviral drugs. In general, antiviral drugs exhibit some beneficial effects in about 20%-30% of treated patients. However, IFNs

are costly and a significant proportion of patients with CHB are not eligible for treatment due to various reasons. On the other hand, lamivudine may need life-long usage. Treatment with lamivudine gives rise to emergence of mutant HBV and breakthrough hepatitis in considerable numbers of patients with CHB[2,3]. These problems related to currently available anti-viral therapies necessitate the development of alternative therapeutic approaches for chronic HBV carriers.

HBV is a non cytopathic virus and the immune responses of the hosts to HBV are important in different aspects of HBV infection. Although the cellular and molecular mechanisms underlying host/HBV interactions are not completely understood, various evidences suggest that host immune responses may have dual role in chronic HBV carriers. In one hand, liver damages in chronic HBV carriers are initiated and maintained by host immune responses. On the other hand, recovery from chronic HBV carrier state is dependent on the intensity and nature of host immune responses[4,5]. Accordingly, induction or suppression of generalized or polyclonal immune responses in HBV carriers is not likely to have therapeutic potentials. In line of this, it has been shown that although chronic HBV carriers exhibit impaired T helper 1 (Th1) responses, Th1-inducing cytokines (interleukin (IL)-12 and IFN-gamma) possess no notable therapeutic effects in these patients[6].

Only recently, some investigators have shown functional differences of HBV-specific immune responses and non HBV-specific immune responses in chronic HBV infection. It appears that products of non HBV-specific immune responses are responsible for liver damages. On the other hand, HBV-specific immune responses possess antiviral potentials without inducing liver damage in chronic HBV carriers[7,8].

Although this indicates that induction of HBV-specific immune responses in chronic HBV carriers may have therapeutic effect, it is extremely difficult to achieve this *in vivo*. Indeed, this is a confusing task. All chronic HBV carriers harbor HBV DNA and all HBV-related antigens. However, they do not exhibit adequate levels of HBV-specific immune responses. Initially, it has been assumed that chronic HBV carriers may be tolerant to some HBV-related antigens such as hepatitis B surface antigen (HBsAg). However, recent studies have revealed that HBsAg-specific immune responses are present in chronic HBV carriers, although the magnitude of immune responses may not adequate to clear or suppress the virus in chronic HBV carriers[9,10]

ROLE OF IMMUNE RESPONSES DURING ACQUISITION AND PROGRESSION OF CHRONIC HBV INFECTION AND ALSO DURING RECOVERY

Two billion people of the world exhibit either present or past infection with HBV and about 400 million of them are chronically infected with HBV. This indicates that less than 20% HBV-infected persons become chronic HBV carriers. It is natural to ask why some persons become chronic HBV carriers, whereas, others control the virus after an acute infection. Again, it is not clear why some chronic HBV carriers do not show any evidence of liver diseases, whereas, others suffer from progressive liver diseases like CH, LC and HCC. Finally, it is elusive why some chronic HBV carriers recover spontaneously. Although the exact cellular and molecular mechanisms underlying these are not completely clear, experimental evidences indicate that viral factor alone do not influence the courses of HBV diseases. Acquisition of HBV carrier state, degrees of liver damages in chronic HBV carriers and recovery from HBV carrier state are not dependent on any particular viral factor such as the amounts of the HBV, genotypes of the HBV, mutations at certain region of the HBV or expression of certain proteins of the HBV[1,2]. Recent investigations have revealed that immune responses of the hosts to HBV may be important in this context (Table 1). Vertical transmission of HBV from HBV-infected mothers (especially hepatitis B e antigen-positive) to neonates usually results in chronic HBV carrier state. This may be related to the concept of 'Neonatal Tolerance' as described by Burnett *et al*[11]. On the other hand, this may be due to impaired functional capacities of neonatal antigen-presenting cells[12].

Table 1 Role of immune responses in chronic HBV carrier

	HBV carriers	Possible causes
A: Acquisition of chronic State	A: Neonates of HBV-carrier mothers B: Immune suppressed Individuals	Inability to induce HBV-specific immunity
B: Induction and Progression of Hepatitis	Chronic hepatitis B	Accumulation of non-specific lymphocytes
C: Recovery from chronic HBV infection	A: Natural recovery	A: Strong and multispecific
	B: Acute hepatitis C: By antiviral drugs	HBV-related immune responses B: Antiviral therapy effective in immune modulatory phase

Some chronic HBV carriers never show any feature of liver diseases during their life time, although they harbor the HBV from their birth day. On the other hand, considerable numbers of chronic HBV carriers exhibit features of necroinflammatory liver diseases. Many of them also suffer from progressive liver diseases such as LC and HCC. HBV is a non-cytopathic virus and it is assumed that HBV-specific HLA-class restricted cytotoxic T lymphocytes (CTLs) act as the principle event determining the intensity of liver damages[13]. However, recent studies indicate that direct destruction of hepatocytes by the CTLs may not the only or principle mechanism of liver damages in patients with CHB. A series of investigations have shown that most of the liver-infiltrating lymphocytes are not HBV-specific. Liver injury could occur as a consequence of large infiltrate of antigen non-specific mononuclear cells. Different types of proinflammatory

cytokines such as IFN-gamma and tumor necrosis factoralpha may be responsible for induction of liver damages because neutralization of these cytokines prevent liver damages in various experimental models of hepatitis[14].

Host immune responses are also important during recovery from chronic HBV carrier state. The HBV-specific immune responses are strong and multispecific in patients with acute hepatitis B who control the virus replication after an acute infection. On the other hand, these are narrowly focused, weak and mono-specific in chronic HBV carriers who are unable to control the virus[13]. This indicates that if proper immune responses against the HBV are initiated after HBV infection, the patients are likely to recover. This is also manifested by the fact that antiviral drugs are mostly effective in immunodulatory phase, but not in immunetolerant phase, of chronic HBV carriers[15].

DEVELOPMENT OF EVIDENCE-BASED IMMUNE THERAPY FOR CHRONIC HBV INFECTION

As described above, immune responses of the hosts to HBV play important roles during acquisition of chronic HBV infection, during progression of liver damages in chronic HBV carriers and also for recovery from chronic HBV carrier state. In this context, it is natural ask what is meant by 'immune response of the host' in chronic HBV infection. This is a very complex issue and this review is not primarily intended to give a detail account of cellular and molecular events related to this. Here, we will focus on HBV-related 'immune responses' those are relevant for developing immune therapy for chronic HBV carriers.

All chronic HBV carriers studied to date, even the immune competent adults who acquire HBV infection, display infrequent, narrowly-focused, and weak HBV-specific immune responses[13]. The purpose of immune therapy in patients with chronic HBV infection is aimed at induction of strong HBV-specific immune responses *in vivo*.

It has been assumed that HBV-specific CTLs are primarily responsible for clearance of HBV-infected hepatocytes in chronic HBV carriers. CTLs are thought to destroy HBV-infected hepatocytes by cytolytic mechanisms. However, the overall concept regarding CTL and their role in destroying virus-infected cells has been changed not only in the context of HBV infection but also in other viral infections. First, CTL is not the only effector cells capable of destroying HBV-infected hepatocytes. Rather, other cells of both innate and adaptive immune system such as HBV-specific CD4 T cells, natural killer cells, macrophages, and neutrophils may also regulate replication of HBV and destruction of hepatocytes[14]. The next, CTLs are not only committed to destroy HBV-infected cells by directly interacting with hepatocytes. Rather, one of the major functions of CTL is to produce various types of cytokines. Series of experiments by different investigators have sown that cytokines are able to induce destruction of HBV or reduced replication of HBV by a non cytopathic mechanism[5,14,16].

Moreover, both HBV-specific and non HBV-specific immune responses are seen in patients with CHB. Several investigators have shown that HBV-specific immune responses are needed for control of HBV replication. On the other hand, non HBV-specific immune responses may induce destruction of hepatocytes. Patients with CHB express high levels of proinflammatory cytokines and accumulation of mononuclear cells in the liver. Recently, it has been shown that most of these mononuclear cells are non HBV-specific[17,18].

Taken together, these features indicate that induction of immune responses is not the main target of immune therapy for chronic HBV carriers. Rather, induction and maintenance of HBV-specific immune responses may have therapeutic implications. The challenging issue is to accomplish this in patients with chronic HBV infection.

VACCINE THERAPY: INDUCTION OF HBV-SPECIFIC IMMUNE RESPONSES

As an approach to induce HBV-specific immune responses in chronic HBV carriers, vaccines containing HBsAg and/or other HBV-related antigens have been administered in patients with CHB. Dienstag *et al* have used hepatitis B vaccine in patients with CHB in early 1980s, however, that study has not been regarded as vaccine therapy because the purpose of that investigation was to evaluate induction of anti-HBs in chronic HBV carriers[19]. The first clinical trial of vaccine therapy was done by Pol *et al* in 1994[20]. Subsequently, clinical trials of vaccine therapy have been performed during last one decade (Table 2)[21-27]. Most of these studies were pilot studies. The present regimen of vaccine therapy has been well tolerated in patients with CHB. It has caused reduced HBV replication, seroconversion to anti-HBe and normalization of serum transaminases in some patients with CHB. However, there is no standard protocol of vaccine therapy. Vaccine therapy has mainly been conducted as pilot studies. All investigators have used their own protocol to perform vaccine therapy. Variable doses of HBsAg (10-20 micrograms) have been used. In some clinical trials, only HBsAg-containing vaccines were used, whereas, in others vaccines containing HBsAg plus preS1 and preS2 proteins were used. The numbers of immunization also varied among studies (3-12 times). Most importantly, there are no standard criteria to assess the response of vaccine therapy.

CAN VACCINE THERAPY STAND THE TASTE OF TIME?

The present regimen of vaccine therapy is unlikely to be an independent therapeutic approach for patients with CHB. A lot of works must be done regarding vaccine therapy. There should be randomized controlled trials of vaccine therapy in different countries. More studies are needed regarding optimum doses of vaccine, compositions of vaccine and numbers of immunization. This therapy is safe for human usage. No side effects of this therapy have yet been published. Vaccines are commercially available and comparatively cheaper. At present, therapeutic efficacy of vaccine therapy is not so satisfactory. But, that is not so important if we consider its future potentials. The concept

of vaccine therapy is unique. Treatment of chronic infection by inducing antigen-specific immune responses deserves attention. There are ample opportunities to improve the present regimen of vaccine therapy.

At present, it appears that the present regimen of vaccine therapy can be used in some specific patients with CHB such as in patients with anti-HBe positive CHB and patients with low levels of HBV replication. The next, vaccine therapy can be used in combination with other antiviral agents. Vaccine therapy can be started after using antiviral drugs for some times in patients with CHB. This may allow better action of this therapy due to decrease of HBV load by antiviral drugs. Again, vaccine therapy can be used first and then antiviral agents can be applied after induction of host immune responses by vaccine therapy. This may be manifested in better therapeutic potential of antiviral agents in CHB patients. We have recently reported a combination therapy of lamivudine and vaccine therapy in patients with CHB. All patients receiving combination therapy became negative for HBV DNA in the sera, whereas, only 56% patients with lamivudine monotherapy lost HBV DNA in the sera within 12 mo. Also, patients with combination therapy did not develop HBV DNA mutation or breakthrough hepatitis[28].

Table 2 Vaccine therapy in patients with chronic hepatitis B. The criteria of response to this therapy have been determined by individual investigators

Protocol	Response	Reference
HBsAg/pre S2, 3 times	Responders: 12 of 32 (38%)	Pol *et al*[20]
HBsAg/anti-HBs vaccine; 3 times	Responders: 9 of 15 (60%)	Wen *et al*[21]
PreS2/S, 3 times (controlled trial)	Responders: 18.8%	Pol *et al*[22]
HBsAg; 12 times	Responders (1 yr: 55%, 2 yr: 77%)	Horiike *et al*[23]
PreS2 vaccine; 3 times	Responders: 60%	Senturk *et al*[24]
S/pre S2; 6 times	Decreased HBV DNA	Ren *et al*[25]
HBsAg/preS2; 3 times	Immune tolerant phage: no significant change	Dikici *et al*[26]
PreS2; 3 times	anti-HBs; 3 of 31 cases	Yalcin *et al*[27]
Combination of lamivudine and HB vaccine	HBV DNA negativation in all 15 cases at 1 yr	Horiike *et al*[28]

DEVELOPMENT OF NEXT GENERATION VACCINE FOR TREATMENT OF CHRONIC HBV INFECTION

Vaccine therapy in patients with CHB was started without adequate discussions regarding its scientific validity and utility. Vaccine therapy was done without paying full attention to common consensuses of immunology and virology. It was surprising to most clinicians why administration of vaccine containing HBsAg in CHB patients will have therapeutic effect because all CHB patients have abundant amounts of HBsAg in the sera and the liver. Moreover, it has been assumed that HBsAg is a tolerogenic antigen in CHB patients. Also the mechanism of action of vaccine therapy in patients with CHB has not been properly analyzed. In general, new therapeutic approaches are usually tested in animal models of human diseases and then these are applied in patients. Vaccine therapy is an exceptional type of therapy. It was first done in patients with CHB without performing preclinical trials in animal models of chronic HBV infection.

In fact, there has been no good animal model of chronic HBV infection. Natural infection of the HBV is limited to some animals only. Lack of an animal model was a major limitation to study immune pathogenesis of chronic HBV infection. During 1980s, HBV transgenic mice (HBV-TM) were produced by microinjecting HBV genome into fertilized eggs of mice[29]. Naturally, there are many differences between HBV-TM and patients with CHB, but HBV-TM represent a suitable immunological animal model of HBV infection. We and others used HBV-TM to develop insights about HBV immunopathogenesis to study various cellular events those can not be investigated directly in human[29,30].

We used a line of HBV-TM that expressed HBsAg and HBV DNA in the sera. This HBV-TM also expressed HBV-related mRNAs in the liver[31]. HBV-TM did not express antibody to HBsAg (anti-HBs) and HBsAg-specific lymphocytes were not detected in HBV-TM. We showed that HBV-TM were unresponsiveness to HBsAg due to impaired function of antigen-presenting dendritic cells (DCs)[32]. Moreover, the immune response defects of HBV-TM were over come by activating endogenous DCs of HBV-TM[33,34]. Next, four controlled trials of vaccine therapy were conducted in HBV-TM. HBV-TM were immunized with vaccine containing HBsAg once in a month for 12 times. It was possible to induce both HBsAg-specific humoral and cellular immune responses in HBV-TM by administration of vaccine containing HBsAg[35-38]. Study on the mechanism of action of vaccine therapy revealed that vaccine therapy induced HBsAg-specific immune responses by activating autologous DCs[37]. Also, the functions of DCs prior to start of therapy had prognostic value during vaccine therapy[38].

DEVELOPMENT OF HBSAG-PULSED DC

DCs are essential for induction and maintenance of antigen-specific specific immune responses. DCs recognize antigens at the site of their localization. They internalize antigens and process them at their endosomal compartments. This leads to expression of antigenic peptides at the surface of DCs. These DCs migrate to lymphoid tissues and activate lymphocytes to become either CTL or CD4+ T cells or antibody-producing B cells[39,40].

During vaccine therapy in HBV-TM, vaccine containing HBsAg was administered to HBV carriers to induce HBsAg-specific immune responses. This was done with the assumption that DCs of HBV-TM will recognize injected HBsAg. Subsequently, HBsAg would be captured, internalized, and processed by DCs of HBV-TM. It was assumed that DCs would activate lymphocytes to induce HBsAg-specific immune responses (Figure 1). But, DCs of HBV-TM had impaired functional capacities and thus endogenous DCs of HBV-TM are not potent inducer of HBsAg-specific immune responses.

We postulated that a powerful regimen of vaccine therapy can be developed by loading DCs with HBsAg in vitro (HBsAg-pulsed DCs). If HBsAg-pulsed DCs exhibit markers of activation and maturation, HBsAg-pulsed DCs will be able to directly induce HBsAg-specific immune responses without any influence of endogenous DCs of chronic HBV carriers. Accordingly, we cultured murine spleen DCs and HBsAg together to produce HBsAg-pulsed DCs. HBsAg-pulsed DCs were more immunogenic than unpulsed DCs. Only two injections of HBsAg-pulsed DCs caused negativity of HBsAg from HBV-TM (Figure 2). HBV-TM injected with HBsAg-pulsed DCs also developed anti-HBs in the sera[41]. Administration of HBsAg-pulsed DCs also induced anti-HBs in immunosuppressed HBV-TM [42, 43].

TRANSLATION OF IMMUNOLOGICAL KNOWLEDGE FROM THE BENCHES TO PATIENT'S BEDSIDES

Wang *et al*. have first reported about functional impairment of DCs in patients with CHB[44]. Subsequently, we localized HBV DNA and HBV RNA in human blood DCs and also showed low IL-12 production capacity of DCs from CHB patients[45]. Similar data regarding functional defects of DCs of chronic HBV carriers have been reported by other investigators during the last 3 year[46,47]. These studies provided the scientific basis for developing DC-based therapy for patients with CHB.

We followed a very careful protocol to produce HBsAg-pulsed blood DCs for human usage[48]. DCs were enriched from human peripheral blood and cultured with HBsAg to prepare HBsAg-pulsed DCs. We used commercial vaccine as a source of HBsAg. The functional capacities of HBsAg-pulsed DCs were assessed *in vitro*. Next, HBsAg-pulsed DCs were administered to human volunteers. There was no features of inflammation, abnormal liver and kidney functions in any volunteers. No volunteer showed any features of autoantibody or autoimmune diseases. Administration of HBsAg-pulsed DCs induced anti-HBs antibody in all volunteers, although the levels of antibody varied among volunteers.

Then, a study was conducted to assess the safety of HBsAg-pulsed DCs in patients with CHB. We prepared DCs from peripheral blood of patients with CHB. Human blood DCs were cultured with HBsAg in commercial vaccine to prepare HBsAg-pulsed DCs. HBsAg-pulsed DCs were administered to patients with CHB. No adverse effects were detected in these patients. Recently, a group of researcher from China has used HBsAg-pulsed DCs for treating patients with CHB[49]. The data of their study is highly encouraging because HBsAg-pulsed DCs exhibited both antiviral and immune modulatory capacities in some patients with CHB. Moreover, they have reported that patients of CHB with normal transaminase levels may also be benefited from this therapy. Although the data of this study is highly stimulating, more information is necessary about the source of HBsAg and characterization of HBsAg-pulsed DCs. Also, detailed data about study protocol will allow undertaking more clinical trials of these therapeutic approaches.

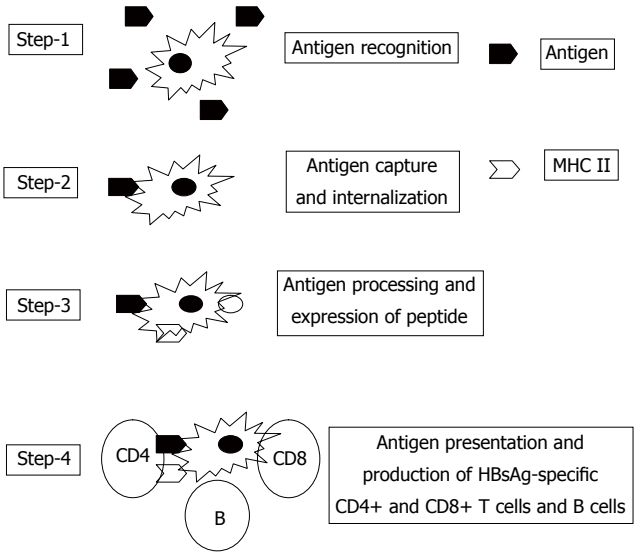


Figure 1 DC/antigen interactions have been shown in 4 steps. Three of these steps are accomplished *in vitro* (step 1-3) when HBsAg-pulsed DCs are prepared.

PRESENT AND FUTURE OF IMMUNE THERAPY AGAINST CHRONIC HBV INFECTION WITH AN EMPHASIS OF IT'S UTILITY IN DEVELOPING NATIONS

The life cycle of HBV, presence of cccDNA of HBV at hepatocytes, and existence of HBV at different extrahepatic sites are major obstacles for eliminating this virus from chronic HBV carriers. It is now apparent that complete eradication of the HBV from chronic HBV carriers may be an unachievable goal[2].

It is also evident that there should be different strategies of HBV control program in developed and developing nations of the world. Less than 10% of total HBV carriers live in developed countries. Moreover, both vertical and horizontal transmissions of HBV have effectively been controlled in most of these countries.

On the other hand, the health care delivery system and economic conditions of developing nations are completely different from those of developed nations, where more than 90% chronic HBV carriers reside. The public health measures are not well developed in most developing nations. Contaminated needles and syringes are used in many facilities, especially in rural areas. There are many chronic HBV carriers among professional blood donors. The numbers of drug abusers are increasing in many of these courtiers. Thus, the numbers of HBV carriers will remain unchanged or increase in most developing nations. China alone harbors about 130 million HBV carriers. Also, there are several million HBV carriers in India.

Regarding treatment, the medical infrastructures of most developed nations allow usage of costly antiviral drugs, regular follow up of treated patients, proper handling of complications and adequate approaches to fight against the mutant HBV. Newer nucleoside analogues are coming to the market. In future, combination therapy with different nucleoside analogues will mainly be used

in patients with CHB in most developed countries. In developed countries, national liver and gastroenterology associations update therapeutic regimens for chronic HBV infection from time to time.

However, usage of antiviral drugs in patients with chronic HBV infection and proper follow up of these patients are difficult in developing nations. IFNs can be afforded by a very small percentage of patients with CHB in these countries. Most of them are not aware of limitation of antiviral drugs in chronic HBV infection. It is generally told that antiviral drugs are effective in about 30% patients with CHB. However, antiviral drugs are recommended for only 10% patients with chronic HBV infection. These drugs are not recommended for majority of chronic HBV carriers. In most developing nations, there is no appropriate system to follow emergence of mutant HBV following lamivudine therapy.

Vaccine therapy in patients with CHB was first conducted in 1994. However, few randomized controlled trials of vaccine therapy have been conducted till now. This situation is different regarding clinical trials of antiviral drugs in developed countries. Antiviral drugs are developed by multinational companies. In contrary, vaccine therapy for chronic HBV carriers has been developed by scientists. This is a cheap therapeutic option for patients with CHB. It is also safe. Vaccine therapy may be popularized if data of controlled trials show their real potentialities. There should be randomized controlled trials of vaccine therapy in patients with CHB in developing countries.

One of the main purposes of immune therapy is to achieve what antiviral drugs could not accomplish in most chronic HBV carriers. The potentiality of vaccine therapy for treating chronic HBV carriers lies in its concept, not in its present ways of application. Truly speaking, this therapy is still in its infancy. Several clinical and experimental studies should be done to optimize protocols of vaccine therapy. At present, commercial vaccines contain HBsAg and/or other components of surface antigens such as preS and preS2 antigens are used in vaccine therapy against chronic HBV carriers. Vaccines containing hepatitis B core antigen or polymerase antigen can also be used in future for treating patients with CHB. However, the main problem is to get safe and human consumable forms of these antigens.

Till now, antigen-pulsed DCs have been used almost exclusively in patients with cancers. Although there are ample opportunities of using antigen-pulsed DCs in chronic infection, allergy and autoimmunity, very few investigators have tried to materialize this in nonmalignant patients. However, we have learnt many important lesions from DC-based therapy in patients with cancers. In the context of DC-based therapy, there is a need to get proper antigens. The next is about the method of preparation of immunogenic antigen-pulsed DCs. Culture of DCs and antigens may lead to production of immunogenic as well as tolerogenic DCs. Accordingly, it is needed to check the nature of antigen-pulsed DCs before administration those to patients. Again, safety is a major concern when antigen-pulsed DCs are administered to patients

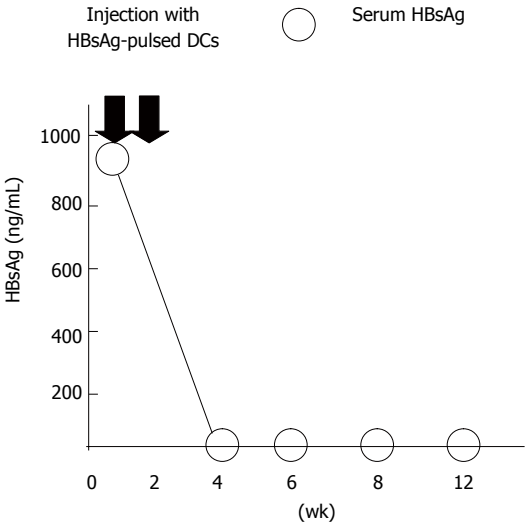


Figure 2 Potent immune modulatory capacity of HBsAg-pulsed DCs in HBV transgenic mice. HBV transgenic mice expressed HBsAg in the sera but not anti-HBs. After two injections of HBsAg-pulsed DCs, these mice became negative for HBsAg in the sera.

with chronic HBV infection. In contrast to patients with cancers, patients with chronic HBV infection are immune competent. Administration of antigen-pulsed DCs may induce autoimmunity in these patients.

At present, clinical trials have been conducting with HBsAg-pulsed DCs. In future, hepatitis B core antigen-pulsed DCs may be used in patients with CHB. The major limitation is to get human grade HBV-related antigens. We used commercial vaccine containing HBsAg to pulse DCs with HBsAg. Chen *et al*[49] have used HBsAg-pulsed DCs in patients with CHB, but they have not mention about the source of HBsAg. The availability of GMP standard HBsAg and other HBV-related antigens will contribute significantly for conducting more clinical trials with antigen-pulsed DCs in patients with CHB. A major challenge will be to achieve GMP standard in developing nations for conducting cell-based therapies including DC-based therapy. This issue can be solved by establishing scientific collaboration among developing countries, developed countries and international agency like World Health Organization.

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REVIEW

Non-steroidal anti-inflammatory drugs in prevention of gastric cancer

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) including cyclooxygenase 2 (COX-2) selective inhibitors, are potential agents for the chemoprevention of gastric cancer. Epidemiological and experimental studies have shown that NSAID use is associated with a reduced risk of gastric cancer although many questions remain unanswered such as the optimal dose and duration of treatment. The possible mechanisms for the suppressor effect of NSAIDs on carcinogenesis are the ability to induce apoptosis in epithelial cells and regulation of angiogenesis. Both COX-dependent and COX-independent pathways have a role in the biological activity of NSAIDs. Knowledge of how NSAIDs prevent neoplastic growth will greatly aid the design of better chemopreventive drugs and novel treatments for gastric cancer.

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Key Words: Nonsteroidal anti-inflammatory drugs; Gastric cancer; Cyclooxygenase; Prevention; Intervention

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INTRODUCTION

Gastric cancer is a major cause of death in the world. However, the etiology of gastric cancer is not fully understood. Although early diagnosis and treatment of gastric cancer significantly improve prognosis[1,2], the 5-year survival rate is only 10%-15% in those with advanced disease[3]. Therefore, the primary prevention of gastric cancer is of particular importance. Several studies have shown that human gastric carcinoma expresses significantly higher levels of cyclooxygenase (COX)-2 than non-cancerous adjacent mucosa[4], suggesting that COX-2 plays a possible role in gastric carcinogenesis. Numerous observations and clinical studies have suggested that the use of NSAIDs is associated with reduced incidence of gastric cancer[5-8]. NSAIDs, particularly the highly selective COX-2 inhibitors, are promising chemopreventive agents[9-12].

Although the effects of NSAIDs on gastric cancer remain unclear, both COX-dependent and independent mechanisms may have roles in the biological activity of NSAIDs. In this review, we concentrate on the use of NSAIDs for gastric cancer prevention and the underlying mechanisms. Furthermore, we also suggest research opportunities on clinical application of NSAIDs for gastric cancer prevention.

NSAIDS AND GASTRIC CANCER

Epidemiological evidence

There are indications, although still limited and preliminary, of a protective effect of NSAIDs on gastric cancers. The risk of gastric cancer is reduced, though not significantly, in the Swedish[13] cohorts of patients with rheumatoid arthritis, with the relative risk (RR) of 0.63 (95%CI = 0.5 to 0.9) for NSAID users. In the American Cancer Society cohort, there is a significant reduction in the risk of death for gastric cancer in aspirin users (RR = 0.64, 95%CI = 0.51 to 0.80). In addition, a dose-dependent trend in risk reduction has been observed, with the RR of 0.73 (95%CI = 0.56 to 0.96) for occasional use (1-15times per month) and 0.49 (95%CI = 0.33 to 0.74) for use 16 or more times per month[5]. With regard to case-control studies, in an American study of cancers of the esophagus and stomach, based on 261 adenocarcinomas of the cardia, 368 noncardia and 695 population controls, current aspirin users have a lower risk for noncardia cancer (RR = 0.46, 95%CI = 0.31 to 0.68), but not for cardia cancer (RR = 0.80, 95%CI = 0.54 to 1.19) when compared to never users. However, there is no significant trend for the risk reduction of gastric cancer with the frequency and the duration of aspirin use[6]. A case-control study from Russia was conducted on 448 gastric cancer cases and 610 hospital controls. The use of aspirin is significantly associated with a risk reduction for the development of gastric cancer (RR = 0.49, 95%CI = 0.31 to 0.77), although the favorable effect seems to be limited for patients with noncardia cancer and *Helicobacter pylori* (*H pylori*) infection[7]. Another case-control study from Sweden, conducted on 567 cases

and 1165 hospital controls, reported that users of aspirin have a moderately reduced risk of gastric cancer compared to never users (RR = 0.7, 95% CI: 0.6 to 1.0), and gastric cancer risk falls with the increasing frequency of aspirin use. Moreover, the risk reduction is statistically significant not only for cardia but also for noncardia cancer, and is slightly more marked in patients with *H pylori* infection[8].

However, there are several limitations for the above observational studies. First, the small number of cases may result in inadequate statistical power[7,8,15]. Second, the choice of control subjects in case-control studies may distort the results because hospital-based controls can not represent population-based controls[16]. Third, different definition of drug exposure and different doses of drugs have been evaluated in these studies. Therefore, the optimal duration and dose of treatment could not be recommended from these studies.

A recent systematic review with meta-analysis of nine observational studies evaluating the association between NSAID use and the risk of gastric cancer suggested that long-term use of aspirin or other NSAIDs is associated with a statistically significant, dose-dependent reduction in the risk of gastric cancer (OR = 0.78, 95%CI = 0.69 to 0.87). When the analysis is stratified by the site of gastric cancer, use of NSAIDs is associated with a statistically significant lower risk for noncardia gastric cancer (OR = 0.72, 95%CI = 0.58 to 0.89), but not for gastric cancer at the cardia. However, whether the minimum effective duration of NSAID use can reduce gastric cancer risk remains undetermined because of the relatively small number of studies, the use of different cut-off time points of treatment duration, and varying definitions of drug exposure[14]. More prospective studies are needed to clarify whether a duration effect exists in the relationship between NSAID use and gastric cancer risk.

Animal models and in vitro studies

The results obtained from animal models evaluating the effect of NSAIDs on gastric cancer have been conflicting. Two studies on animal models of carcinogens suggested that NSAIDs (aspirin and flurbiprofen) could enhance chemical-induced gastric carcinogenesis in rats[17,18], indicating that prostglandins (PGs) produced by the gastric mucosa protect against the action of carcinogens in this rodent model. In contrast, broad anticarcinogenic effects of NSAIDs have been demonstrated both *in vivo* using laboratory animals and *in vitro* using cancer cell lines. Chemically-induced gastric tumors can be reduced by aspirin[19], indomethacin[20], sulindac and ibuprofen[21] in mice. Sawaoka *et al*[22] have investigated the effects of NS-398, a selective COX-2 inhibitor, or indomethacin, a non-selective COX-2 inhibitor, on the growth of gastric cancer xenografts *in vivo*. Both drugs could reduce the tumor volume significantly in a dose-dependent manner. Fu *et al*[23] also found that sulindac and celecoxib obviously decrease the blood vessel quantity and tumor size of SGC7901 cancer xenografts. In a recent study Hu *et al*[24] showed that treatment with celecoxib could suppress MNNG-induced gastric cancer in rats. Indomethacin, sulindac and NS-398 significantly inhibit the proliferation and growth of human gastric cancer cell line MKN28 *in vitro*[25,26].

POSSIBLE MECHANISMS OF CANCER PREVENTION

The results described above provide convincing evidence that non-selective NSAIDs and selective COX-2 inhibitors can inhibit gastric cancer cell formation and growth. However, the molecular basis for the striking chemo-preventive effects has not been fully clarified. Several studies indicate that COX-2 expression is a relatively early event during carcinogenesis in the stomach[27-30]. The best known target of NSAIDs is COX, the rate limiting enzyme in the conversion of arachidonic acid to prostanoids. Thus, the chemo-preventive effects of NSAIDs on gastric cancer may be attributed to inhibition of COX and the resulting decrease in production of PGs[31-33]. However, there are several studies that conflict with this observation. For example, NSAID derivates that lack the ability to inhibit COX (sulindac sulfone) have been shown to inhibit gastrointestinal tumor growth *in vivo* and *in vitro*[34-37]. COX-1 and COX-2 null mouse embryofibroblast cells remain sensitive to the anti-proliferative and anti-neoplastic effects of NSAIDs[38]. Furthermore, the concentration of NSAIDs that inhibits the growth of cancer cells *in vitro* is 10-100 times higher than that required to inhibit COX activity, suggesting the existence of additional cellular targets.

Induction of apoptosis

A substantial body of evidence now supports the idea that the induction of programmed cell death (apoptosis) is one of the main ways by which NSAIDs prevent cancer in a COX-2 dependent manner or a COX-2 independent manner.

COX 2-dependent mechanisms

One of the primary pharmacological properties of the NSAIDs is their ability to inhibit the COX. Analysis of COX expression has shown that COX-2 is increased in gastric cancer, but not in normal mucosa[4,39-42]. These findings support the idea that COX-2 over-expression is important during gastric carcinogenesis. In addition, over-expression of COX-2 can increase production of PGE2, adhesion to the extra-cellular matrix, concentrations of BCL2, and reduce TGF-2 receptor expression and E-cadherin protein. All these changes, which suggest an increased tumorigenic potential, support the notion that COX-2 overexpression alters the biology of cells and plays a part in the transformation process[43,44]. The question of how COX-2 inhibition induces apoptosis has been hotly debated. Several studies have suggested that decreased COX-2 activity can reduce eicosanoids such as the PGs, and in turn affect cell proliferation and apoptosis[45]. So far, there is no definitive evidence that supports the existence of a signaling pathway through which PGs can directly affect apoptosis. However, specific observations provide some clues and warrant mention here. The peroxisome proliferator-activated receptors (PPARs), a family of ligand-activated transcription factors comprising a subset within the steroid receptor family of intracellular receptors, have been shown to modulate the apoptotic response of various cell types[46,47]. It is conceivable that certain COX-2

products may be physiological ligands for the PPARs. Thus, it is hypothesized that PPARs are downstream of COX-2 in gastric carcinogenesis and may play a part in NSAIDs-mediated antineoplastic effects[48-50].

Arachidonic acid may also provide a mechanism underlying COX-2-dependent NSAIDs-induced apoptosis. Arachidonic acid is produced by hydrolysis of phospholipids and triglycerides. The phospholipase A2 cleaves phospholipids and releases arachidonic acid into the cytosol. Once released, it serves as a precursor for PGs production and is a signaling molecule in its own right. Treatment with various NSAIDs results in inhibition of COX-2 and a dramatic increase in the concentration of arachidonic acid, the main substrate for COX-2[45,51]. This buildup of arachidonic acid stimulates the sphingomyelinase to convert sphingomyelin to ceramide, a potent inducer of apoptosis[52-55]. Other studies have shown that the cellular concentration of arachidonic acid is a general mechanism by which apoptosis is regulated[56-59]. It seems that arachidonic acid can alter mitochondrial permeability and cause cytochrome C release, leading to apoptosis[58,60].

COX 2-independent mechanisms

Several recent observations cast doubt on the idea that COX is the sole target of NSAID action. The finding that some NSAIDs can inhibit proliferation and induce cell death in cells that do not express COX, suggests that other targets may play a part in NSAIDs-mediated apoptosis[61].

One potential mechanism involves the transcription factor NF-κB, which promotes cell survival and enhances proliferation. Several investigators have suggested that NSAIDs could promote apoptosis by inhibiting NF-κB signaling pathway[62-64]. The effect that seems to be due to its ability to inhibit the activity of IκB kinase β (IKKβ), an enzyme that activates the NF-κB pathway by phosphorylating the inhibitory subunit of NF-κB (IκB-α) and targeting it for destruction. Subsequently, genes required for cancer cell growth and survival may not be transcribed[65]. Furthermore, Yasui *et al*[66] reported that sulindac inhibits TNF-α-mediated NF-κB activation and greatly sensitizes human gastric cancer cell line MKN45 to TNF-α. The study data strongly suggest that combination therapy of TNF-alpha with sulindac may sensitize tumor cells to TNF-α and augment its proapoptotic potential. Moreover, Wong *et al*[67] showed that SC236, a selective COX-2 inhibitor, could inhibit NF-κB mediated gene transcription and binding activity in gastric cancer. This effect occurs through a mechanism independent of COX activity and PG synthesis. Furthermore, unlike aspirin, SC236 affects neither the phosphorylation, degradation, nor the expression of IκB-α, suggesting that the effects of SC236 are independent of IKK activity and IκB-α gene transcription. Instead, SC236 works directly through suppressing the nuclear translocation of RelA/p65. It is possible that SC236 directly targets proteins that facilitate the nuclear translocation of NF-κB[67].

The role of protein kinase C (PKC) and apoptosis-related oncogenes has also attracted more attention. Zhu *et al*[68] showed that both aspirin and indomethacin induce apoptosis in gastric cancer cells which may be mediated by up-regulation of *c-myc* proto-oncogene. PKC activation can abrogate the effects of NSAIDs by decreasing *c-myc* expression. Jiang *et al*[69] also showed that SC-236 induces apoptosis in AGS cells. SC-236 down-regulates the protein expression and kinase activity of PKC-β(1) in AGS cells, which provides an explanation for COX-independent apoptotic effects of selective COX-2 inhibitors in cultured gastric cancer cells. They also suggested that PKC-β(1) acts as a survival mediator in gastric cancer. Down-regulation of PKC-β(1) by SC-236 may provide new targets for future treatment of gastric cancer[69].

Furthermore, it was reported that the pro-apoptotic proteins Bax and Bak play a role in NSAIDs-induced gastric cancer cell apoptosis[70], suggesting that one of the major pathways mediating the anti-tumor response of aspirin and indomethacin in gastric cancer cells is through the up-regulation of Bax and Bak and activation of caspase-3.

Besides, Wu *et al*[71] demonstrated that SC-236 induces apoptosis in gastric cancer cells via the up-regulation of 15-lipoxygenase-1 (15-LOX-1), and increases endogenous 13-S-hydroxyoctadecadienoic acid (13-S-HODE), a novel target for chemoprevention effect of NSAIDs.

Regulation of angiogenesis

It is now clear that angiogenesis is critical for the progression of most human cancers[72]. Angiogenesis is a complex process controlled by a balance of angiogenic and angiostatic factors involved in multiple pathways that result in endothelial cell proliferation, differentiation, and organization into a functional network of vascular channels. Among the reported angiogenic factors, vascular endothelial growth factor (VEGF) is the most powerful endothelial cell specific mitogen that plays a key role in the complicated process of angiogenesis.

COX-2 has been shown to directly promote angiogenesis in several different experimental systems[73,74]. Masferrer *et al*[80] have examined more than 150 samples of different types of human cancers and found that COX-2 is present in the angiogenic vasculature in most of the tumors analyzed. Therefore, COX-2-derived PGs contribute to tumor growth by inducing newly formed blood vessels that sustain tumor cell viability and growth. Furthermore, xenografts of COX-2 over-expressing gastric cancer, when implanted into nude mice, express angiogenic factors in a COX-2-dependent manner, and both angiogenesis and tumor growth can be inhibited in these mice by administration of NSAIDs[23,78].

COX-2 expression has been shown to correlate with microvessel density (MVD), an accepted histologic marker of tumor angiogenesis in several different human malignancies, including gastric cancer[30]. Recent years, the relationships between COX-2 expression, VEGF expression, tumor angiogenesis, and outcome parameters in gastric cancer patients have been reported[75-77]: COX-2 expression significantly correlates with VEGF, and the colocalization of these proteins is most frequent at the advancing edge of cancer cells[75]. MVD is higher in COX-2 and VEGF positive cases than in COX-2 and VEGF negative cases[76]. Co-expression of COX-2 and VEGF, age, lymph node status, and serosal invasion are

independent prognostic factors for overall survival in gastric cancer patients[77].

Interestingly, COX-1, the other isozyme expressed in tumor vascular endothelia, participates in tumor angiogenesis, because an anti-sense oligonucleotide of COX-1 suppresses *in vitro* angiogenesis induced by COX-2-overexpressing cells. Non-specific COX inhibitors reduce the growth and angiogenesis in cancer xenografts by inhibiting COX-1 in vascular endothelial cells, even when the tumor does not express COX-2. These results demonstrate that COX inhibitors suppress angiogenesis and tumor growth by inhibiting expression of angiogenic factors and vascular endothelial cell migration[79].

COX-2 expression and H pylori infection

H pylori infection is also a strong risk factor for the development of gastric cancer via the pathway of atrophy, intestinal metaplasia and dysplasia in chronic infection. Infection with *H pylori* up-regulates mediators of the inflammatory response, leading to the production of inflammatory cytokines and PGs which themselves may suppress cell-mediated immune responses and promote angiogenesis. These factors may also have effects on cell growth and survival signaling pathways, resulting in induction of cell proliferation and inhibition of apoptosis[81].

H pylori up-regulates COX-2 mRNA expression and PGE_2 synthesis in gastric cancer cells[82], and both pre-malignant and malignant gastric lesions demonstrate high COX-2 expression levels[83]. Therefore, *H pylori*-induced gastric carcinogenesis may be associated with the elevated expression of COX-2 in gastric epithelium. It is possible that NSAIDs may inhibit the replication and proliferation of *H pylori*[84,85], neutralize the increased COX-2 expression and PG synthesis and reverse the increased apoptosis and proliferation of epithelial cells associated with *H pylori* infection[86,89], thereby reducing the risk of gastric cancer[90,91]. Experimental models of cell proliferation have also shown that, in hyperplastic gastritis in the murine model infected with *H pylori*, treatment with etodolac could significantly decrease PGE_2 production and the thickness of gastric pits in the infected groups[87].

Whether eradication of *H pylori* can prevent gastric cancer remains contradictory. COX-2 expression remains in gastric epithelium even after successful eradication of *H pylori*[88]. This may suggest that combined treatment of COX-2 inhibitors and *H pylori* infection may be more useful in eliminating *H pylori*-induced COX-2 expression than either anti-*H pylori* antibiotics or COX-2 inhibitors alone. This issue deserves further study.

CONCLUSIONS AND FUTURE DIRECTIONS

Combining the evidence from humans, animal models and cell culture systems can establish the targeted inhibition of COX-2 was a viable approach to gastric cancer prevention and/or treatment. However, despite these successes, many questions remain unanswered such as the optimal dose and duration of treatment. Selective COX-2 inhibitors, which have an advantage of minimizing the gastrointestinal side-effects, have received wide attention. An exciting area for future investigation is to investigate whether COX-2 inhibitors can be used in the treatment of patients who have preneoplastic gastric lesions or suffer from gastric cancer, and whether combination therapy with COX-2 inhibitors and the drugs targeting other oncogenic pathways can lead to improved clinical outcomes.

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GASTRIC CANCER

Anticancer effect of Jinlongshe granules on *in situ*-transplanted human MKN-45 gastric cancer in nude mice and xenografted sarcoma 180 in Kunming mice and its mechanism

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Abstract

AIM: To study the antitumor effect of Chinese compound Jinlongshe (JLS) granules on sarcoma 180 and MKN-45 human gastric cancer cell lines *in vivo* and its mechanism.

METHODS: After establishment of S180 sarcoma (S180) and MKN-45 gastric cancer model of nude mice, the tumor-bearing mice were divided into 5 groups at random. Three experimental groups were respectively given the aqueous extract of JLS granules at doses of 120 g, 60 g and 20 g /(kg per 6/wk, *i.g*) for 3 wk in S180 and 6 wk in nude mice model. Positive control was given cyclophosphamide (Cy) at a dose of 50 mg/(kg per 3 /wk, *i.g*) for 3 wk in S180 models and 5- Fluorouracil (5-FU) 20 mg/(kg per 3 /wk, *i.g*) for 3 wk in nude mice model. Negative control was given normal saline (NS) at a dose of 0.18 g/(kg per 6/wk, *i.g*) respectively. After 3 wk in mice bearing S180 tumor and 6 wk in nude mice model, the experimental animals were sacrificed and the masses of tumor were weighed, and the rates of tumor inhibition of each treated group were calculated respectively. To determine the antitumor mechanisms, the morphological changes, cell cycle and apoptosis were observed in MKN-45 nude mice model. Annexin V-FITC/PI double staining FCM assay was used to further determine the live cells, apoptotic cells, necrotic cells and debris.

RESULTS: The inhibitory rates of JLS granules at the doses of 20 g/kg, 60 g/kg and 120 g/kg were 50.31%, 55.94% and 68.13% ($P < 0.01$) in nude mice models and 40.90%, 50.32% and 58.46% ($P < 0.01$) in S180 model. The inhibitory rate of Cy was 85.22% in S180 models and the inhibitory rate of 5-FU was 53.43% in nude mice model ($P < 0.01$). Nuclear chromatin and margination were observed under a transmission electron microscope (TEM). The G0/G1 phase was arrested, typical apoptotic peak appeared, the apoptotic rate was 22.81%-38.54% in three JLS granule-treated groups. Annexin V-FITC/PI double staining FCM assay showed that the apoptotic cells were 4.36%, 3.08%and 7.08% in three dosages, most cells were localized in the low right quadrant.

CONCLUSION: Jinlongshe granules possess anti-tumor effects on experimental tumor models *in vivo*, and apoptosis induction is one of its anti-tumor mechanisms.

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Key words: Jinlongshe granules; Sarcoma 180; Human MKN-45 gastric cancer lines; Traditional Chinese medicine

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INTRODUCTION

Gastric cancer is one of the most leading causes of cancer-related deaths worldwide[1]. Despite its decreasing trend in the Western world during the recent two decades, gastric cancer still accounts for about 23.2% of all cancer deaths in China[2-4]. Although surgery, chemotherapy, and irradiation are the mainstream therapeutic methods for gastric cancer, undesirable chemotherapy reaction and poor prognosis in the advanced gastric cancer patients are still the major problem for gastric cancer treatment[5]. Consequently, combination of multi-therapeutic methods may effectively improve gastric cancer patient's symptoms and prolong their survival time[6-7].

Jinlongshe (JLS) granules are an oral Chinese medicine compound, consisting of Chinese herbs (*Rhizome Arisaemat, Rhizoma Pinelliae, corium stomachium galli, Radix*

Glycyrrhizae preparata, etc.), developed by our department based on TCM theories of "phlegm" as the key factor in preventing and treating gastric cancer[8]. However, the exact antitumor mechanism of JLS granules is unclear. This study was to observe the anti-tumor effect of JLS granules and to determine its mechanism using sarcoma 180 and human gastric cancer cell line MKN-45 *in vivo*.

MATERIALS AND METHODS

Experimental animals

BALB/C nude mice (19-22 g) and Kunming mice (20-22 g) were provided by Experimental Animal Center of Chinese Academy of Sciences, Shanghai, China. All experimental animals were housed in specific-pathogen free (SPF) condition for 1 wk to adapt the surroundings before initiation of the experiment and had free access to food and water throughout the study.

Cancer cell lines

S180 cell line and MKN-45 human gastric denocarcinoma cell line were provided by Institute of Pharmaceutical Research, Shanghai, Chinese Academy of Sciences.

Drug samples and reagents

Crude JLS granules were purchased from Leiyunshang Company, Shanghai, China. An aqueous extract of JLS granules at a concentration of 6 g/mL of raw materials was provided by Shanghai Changzheng Hospital. The aqueous extract was diluted with purified water before use. Cyclophosphamide injection (Cy, Lot No. 040909) was produced in Hualian Pharmaceutical Co., Ltd, Shanghai, China. OB Biogel (Lot No.040514) was produced in Baiyun Medical Biogel Co., Ltd, Guangzhou, China. Ketamine hydrochloride injection (Lot No.02072332) was produced in Hengrui Pharmaceutical Co., Ltd, Jiangsu, China.

Establishment of in situ-transplanted human MKN-45 gastric carcinoma nude mouse model

The procedure of *in situ*-transplanted gastric cancer nude mouse model was referred to Xu and Chen Ling *et al*[9-10]. In brief, before transplantation, nude mice bearing gastric denocarcinoma MKN-45 cell lines were killed. Then the tumor was dissected and put into aseptical saline. Pieces of intact, fish meat-like tumor tissue of 1 mm × 1 mm × 1 mm in diameter from margin were prepared after removal of tumor capsule. Mice anesthetized with ketamine (50 mg/kg, ip) were sterilized with caseoiodine and a transverse incision was made on the left upper quadrant. After exposure of the greater curvature of stomach, serosa and muscular layer were incised with needle. Then tumor tissues were transplanted under serosa of stomach and one drop of OB biogel was applied to the tumor tissue. After 40 s, stomach was brought into abdominal cavity and abdominal wall was finally closed. The whole operation procedure was completed following the doctrine of aseptic manipulation.

Establishment of S180 model

This model was by subcutaneous injection as previously described[11]. Briefly, the S180 mouse sarcoma cells with ascites were harvested, diluted with sterilized saline at a ratio of 1:8 (cell concentration was adjusted to 1.0×10^6/mL), and inoculated subcutaneously into the right armpit region of Kunming mice.

Inhibitory effect of JLS granules on S180 and MKN-45 in vivo

Forty-eight hours after establishment of the MKN-45 gastric cancer model and 24 h after establishment of S180 model, the transplanted mice were divided into 5 groups according to the method described in "National Requirements for *in vivo* Screening of Anti-neoplastic Drugs"[12]. The dose of JLS granules was decided according to the clinical dose used in humans. The mice bearing S180 were killed by dislocation of cervical vertebra 3 wk after treatment. The nude mice bearing *in situ*-transplanted MKN-45 gastric cancer cell line were sacrificed 6 wk after treatment. The tumor weights were measured and the inhibitory rates were calculated according to the following equation: rate of inhibition (%) = (mean tumor wt of negative control -mean wt of treated group)/mean tumor wt of negative control ×100.

Morphologic assessment of apoptosis in MKN-45 nude mice

We selected NS group, 5-FU group and 60 g/kg JLS granules group for morphologic assessment of apoptosis in MKN-45 nude mice. Tumor tissue was cut into small pieces, homogenized into 1 mm × 2 mm, prefixed with 4% paraformaldehyde for 4 h and washed with PBS (0.1 mmol/L), post-fixed with 1% osmic acid, dehydrated in graded ethanol-acetonum, embedded in an Epon 812 mixture, and cut into sections on a ultramicrotome. After stained with uranyl acetate and lead citrate, the sections were examined under a Hitachi-800 transmission electron microscope.

Cell cycle and apoptosis in MKN-45 nude mice

Fresh tumor tissue separated from nude mice was chopped with mechanical method. Single cell-suspended solution was obtained with 200 filter screens. The suspensions were divided into two parts. One part was fixed with 70% ice-cold ethanol for at least 12 h at 4 ℃ and then washed with PBS again before examination. The fixed cells were treated with RNase A and stained with PI at 37 ℃ for 30 min. Analysis was performed with a Coulter FCM (EPICS-XL) and data were analyzed with Multicycle DNA content and cell analysis software.

Annexin V-FITC/PI double staining for FCM-assessed apoptosis

The Annexin V FITC kit (Jingmei Company, Lot No. 20050910) was used for detecting apoptosis according to manufacturer's instructions. The suspensions were washed twice and adjusted to a concentration of 1×10^6 cells/mL with ice cold 4 ℃ PBS. The Falcon tubes (12 mm × 75 mm, polystyrene round-bottom) were used in experiment, 100 μL of suspensions was added to each labeled tube, 10 μL of Annexin V-FITC and 10 μL 20 μg/mL PI were added into the labeled tube, incubated for at least 20 min at room temperature in the dark, then 400 μL of PBS

Table 1 Anti-tumor effect of JLS granules on mice bearing S180

Group	*n*	Tumor weight (g)	Inhibition rate (%)
Control	10	4.67 ± 3.27	-
120 g/kg	10	1.94 ± 2.03[ab]	58.46
60 g/kg	10	2.32 ± 2.83[ab]	50.32
20 g/kg	10	2.76 ± 2.32[ab]	40.90
Cy	10	0.69 ± 0.33[b]	85.22

[a]$P < 0.05$ *vs* Cy; [b]$P < 0.01$ *vs* control.

Table 2 Anti-tumor effect of JLS granules on MKN-45 cells in nude mice

Group	*n*	Tumor weight (g)	Inhibition rate (%)
Control	10	3.20 ± 0.96	-
120 g/kg	10	1.02 ± 0.57[b]	68.13
60 g/kg	10	1.41 ± 1.39[b]	55.94
20 g/kg	10	1.59 ± 1.12[b]	50.31
5-FU	8	1.49 ± 1.37[b]	53.43

[b]$P < 0.01$ *vs* control.

binding buffer was added to each tube without washing and analyzed by FCM as soon as possible (within 1 h).

Statistical analysis

Data were presented as mean ± SD and analyzed with SPSS 11.0 software. The analysis of variance (ANOVA) was used and least significant difference *t* test (LSD-*t*) was used for group comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Anti-tumor effect of JLS granules on S180

JLS granule at the doses of 20 g/kg, 60 g/kg and 120 g/kg significantly inhibited the growth of S180 subcutaneous tumor($P < 0.01$) in a dose-dependent manner, but lower than the inhibitory rates of Cy ($P < 0.05$) (Table 1).

Anti-tumor effect of JLS granules on in situ-transplanted gastric cancer MKN-45 cell lines

JLS granule at the doses of 20 g/kg, 60 g/kg and 120 g/kg significantly inhibited the growth of orthotopic-transplanted MKN-45 gastric cancer($P < 0.01$) in a dose-dependent manner, but no difference statistically compared with 5-FU ($P > 0.05$) (Table 2).

Morphology changes

Condensation of chromatin at margins of nuclei, disintegration of nucleolus, vacuoles in cytoplasm were observed in 60 g/kg group and 5-FU also had the same apoptotic appearance under TEM (Figure 1).

Assessment of cell cycle and apoptosis of MKN-45 cells in nude mice

The percentage of G0/G1 cells in JLS granules group was higher than that in NS group and 5-FU group in a dose-dependent manner ($P > 0.05$). The cell percentage of S phase in of JLS group and 5-FU group was lower than that in control group ($P < 0.05$), the apoptotic rate in 120 g/kg JLS group was higher than that in control group, 20 g/kg JLS group and 5-FU group ($P < 0.05$). The typical apoptotic peak was observed (Table 3, Figure 2).

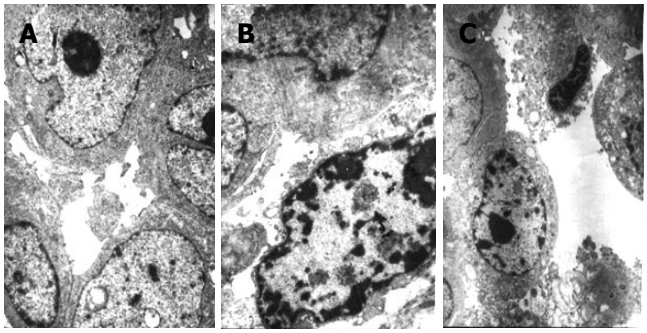


Figure 1 Ultra-structure observed under transmission electron microscopy in control group × 4 000 (**A**), 60 g/kg JLS group × 6 000 (**B**), 5-FU group × 3 000 (**C**).

Table 3 Effect of different treatments on cell cycle and apoptosis of MKN-45 cells (mean ± SD)

Group	*n*	G_0/G_1	G_2/M	S	Apoptotic rate
Control	10	42.40 ± 11.28	21.28 ± 11.43	36.92 ± 12.21	19.88 ± 14.83[c]
120 g/kg	10	50.58 ± 8.78	26.45 ± 12.53	14.56 ± 9.35[a]	38.54 ± 12.65[a]
60 g/kg	10	47.55 ± 7.02	26.51 ± 15.05	19.36 ± 12.69[a]	28.27 ± 11.25
20 g/kg	10	46.37 ± 12.09	25.47 ± 14.79	24.04 ± 13.27[a]	22.81 ± 10.65[c]
5-FU	8	41.78 ± 4.30	23.59 ± 11.97	19.97 ± 13.31[a]	24.97 ± 9.93[c]

[a]$P < 0.05$ *vs* control, [c]$P < 0.05$ *vs* 120 g/kg.

Assessment of apoptosis in MKN-45 nude mice

Results by Annexin V-FITC/PI double staining for FCM assay showed that the early apoptotic cells were 4.36%, 3.08% and 7.08% in the three JLS granules groups (Figure 3), and most of cell populations in 5-FU treated group were located in the up right quadrant and the cells on the lower right quadrant were not many as compared with JLS granules groups.

DISCUSSION

Tumorigenesis, in a sense, is due to the deregulation in cell proliferation and apoptosis[13-14], accordingly tumor treatment not only inhibits cell proliferation, but also induces apoptosis. More and more attention is paid to apoptosis in treatment of tumors and the effectiveness of anti-tumor agents is often evaluated[15,16]. Investigators have confirmed that many Chinese herbs have antitumor property and induction of apoptosis is one of the mechanisms[17-20]. But most of these studies aimed directly at single herbs, bioactive constituents or leading compounds extracted from herbs, and could not educe the multi-target action of Chinese compounds and traditional Chinese medicine (TCM) theories in disease therapy[21].

In TCM theories, "phlegm" is not only a pathologic metabolite, but also a causative agent. The metabolites such as ectopic hormone, growth factor and tumor

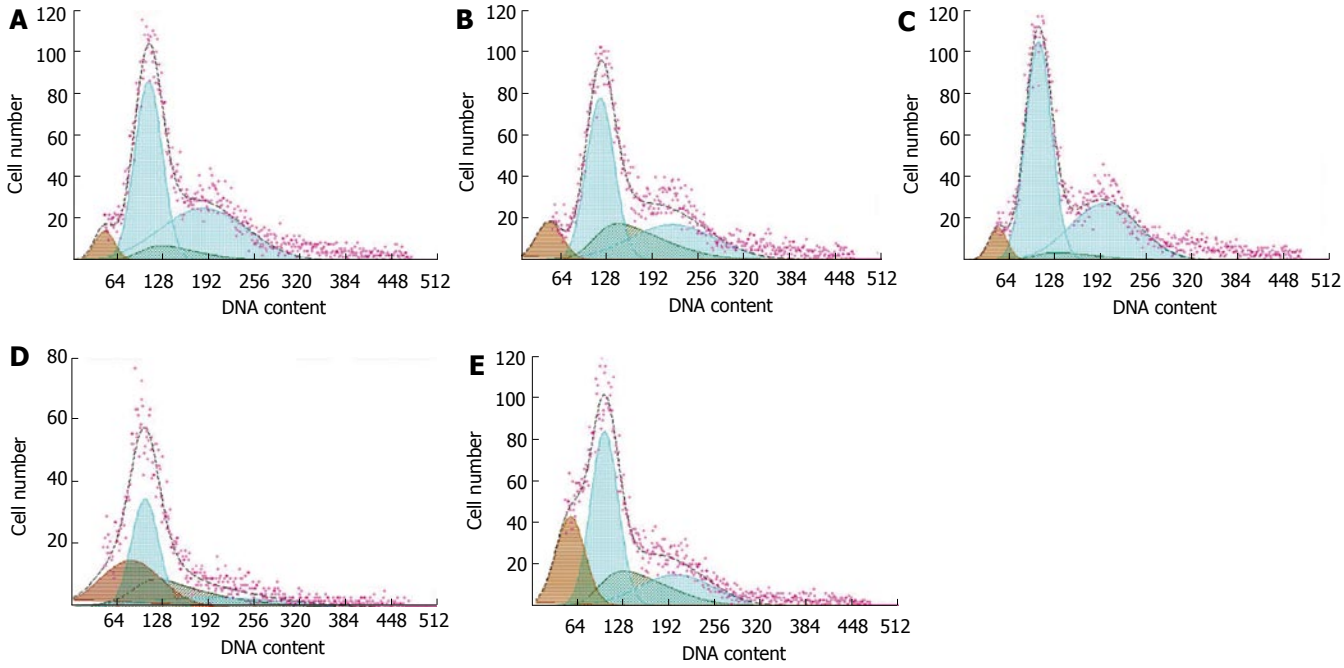


Figure 2 Histogram observed under FCM in 120 g/kg JLS group (**A**), 60 g/kg JLS group (**B**), 20 g/kg JLS group (**C**), control group (**D**), and 5-FU group (**E**).

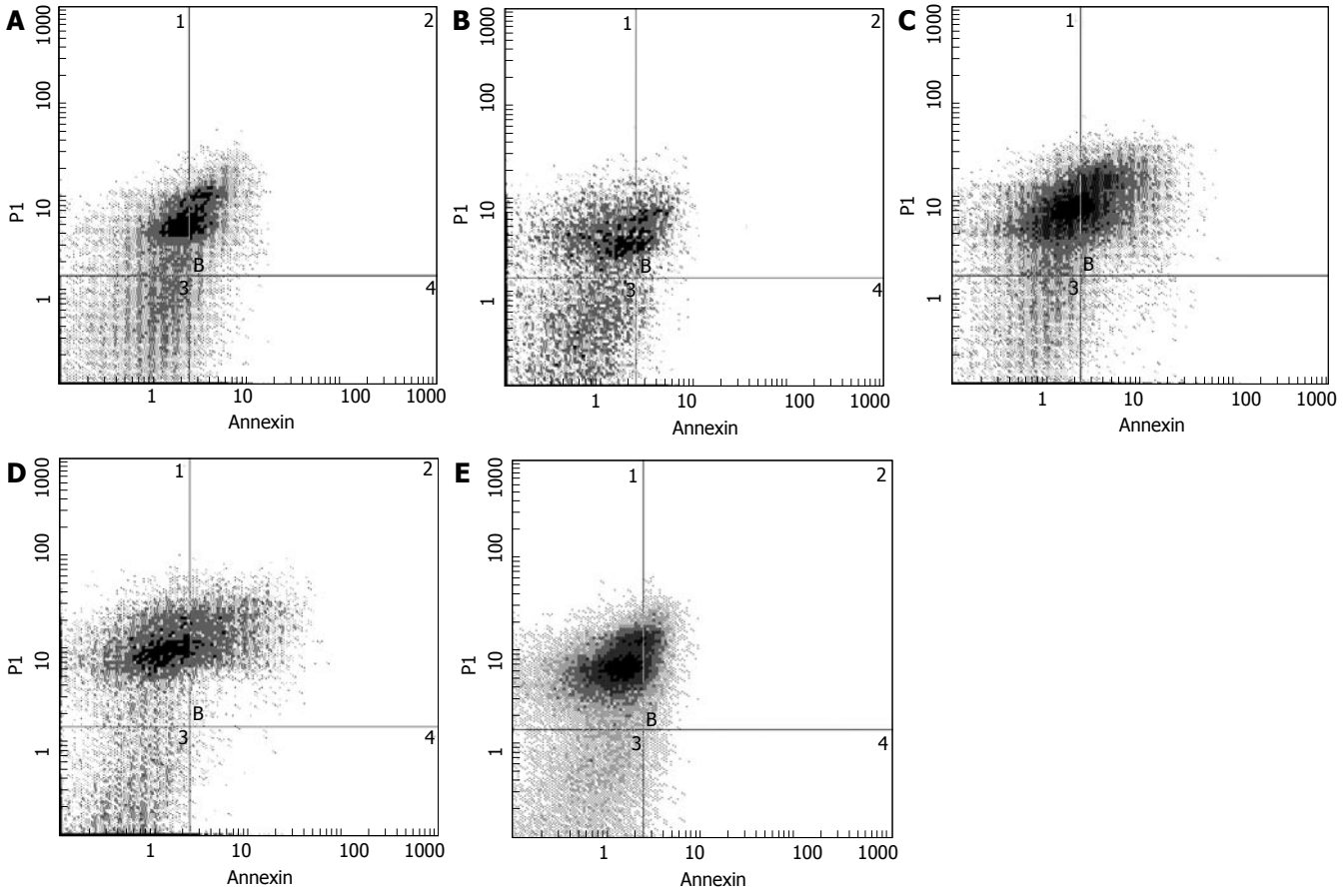


Figure 3 Cytogram observed by Annexin V-FITC/PI double staining assay in 120 g/kg JLS group (**A**), 60 g/kg JLS group (**B**), 20 g/kg JLS group (**C**), control group (**D**), and 5-FU group (**E**).

markers produced by tumor cells all belong to "phlegm", which can devitalize physiologic functions and stimulate tumor invasion and metastasis in turn. Therefore, "dispersing phlegm and eliminating stagnation" (cleaning metabolites produced by tumor) is one of the regimens for tumor treatment in TCM. JLS granules are an anti-

tumor compound prescription developed according to the methods of "dispersing phlegm and eliminating stagnation". Our previous experiments have shown that "dispersing phlegm and eliminating stagnation" inhibit could VEGF, Ras mRNA and protein expression[22-24] and have good antitumor and anti-metastasis potency *in vivo*. However whether it induces apoptosis is unclear. Our studies showed that JLS granules had anti-tumor effects on S180 and MKN-45 human gastric cancer. The ultra-structure of MKN-45 gastric cancer tissue treated with JLS granules showed that there were apoptosis changes. Cell cycle and apoptosis analysis also demonstrated that JLS granules could arrest MKN-45 cells at G0/G1 phase, decrease S phase cell populations. The typical apoptosis peak further confirmed its apoptosis-inducing effect.

In addition, changes in the plasma membrane are one of the earliest morphological features of apoptosis. At this time, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36kD Ca^{2+}-dependent phospholipid binding protein that has a high affinity for PS, thus Annexin V staining serves as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis[25-26]. Therefore, staining with Annexin V in conjunction with vital dyes such as propidiumiodide (PI) can identify early apoptotic cells (Annexin V positive, PI negative). In our study, cells treated with JLS granules were located in the low right quadrant and cells in 5-FU-treated group were located in the upper left quadrant, suggesting that the earlier apoptotic cells in JLS granules-treated groups are higher than those in 5-FU-treated group and that JLS granules induce apoptosis in human gastric cancer MKN-45 cells.

In conclusion, JLS granules have the inhibiting effects on human gastric carcinoma and induction of apoptosis may be one of its mechanisms.

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BASIC RESEARCH

Partial Beclin 1 silencing aggravates doxorubicin- and Fas-induced apoptosis in HepG2 cells

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Abstract

AIM: To investigate the role of Beclin 1 on the susceptibility of HepG2 cells to undergo apoptosis after anti-Fas antibody or doxorubicin treatment.

METHODS: Beclin 1 silencing was achieved using RNA interference. DNA ploidy, the percentage of apoptotic cells and the mitochondrial membrane potential were assessed by flow cytometry. Levels of Beclin 1, Bcl-X_L and cytochrome *c*, and the cleavage of poly (ADP-ribose) polymerase (PARP) were assayed by using Western blots.

RESULTS: Beclin 1 expression decreased by 75% 72 h after Beclin 1 siRNA transfection. Partial Beclin 1 silencing significantly increased the percentage of subG1 cells 24 and 40 h after treatment with doxorubicin or anti-Fas antibody, respectively, and this potentiation was abrogated by treatment with a pan-caspase inhibitor. Partial Beclin 1 silencing also increased PARP cleavage, mitochondrial membrane depolarization and cytosolic cytochrome *c*. The pro-apoptotic consequences of partial Beclin 1 silencing were not associated with a decline in Bcl-X_L expression.

CONCLUSION: Partial Beclin 1 silencing aggravates mitochondrial permeabilization and apoptosis in HepG2 cells treated with an anti-Fas antibody or with doxorubicin.

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Key words: Beclin 1; Apoptosis; HepG2 cells; Anti-Fas antibody; Doxorubicin

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INTRODUCTION

Beclin 1, a recently discovered tumor suppressor gene located on human chromosome 17q21, is mono-allelically deleted in 40%-70% of sporadic mammary, ovarian and prostate tumors[1]. Heterozygous beclin $1^{+/-}$ transgenic mice display a high incidence of spontaneous malignancies, including hepatocellular carcinoma (HCC), although the remaining beclin 1 allele is neither lost, mutated or silenced in these tumors[2,3]. These data have led to the conclusion that beclin 1 is a haploinsufficient tumor suppressor. One mechanism whereby beclin 1 haploinsufficiency can promote cancer is impaired autophagy, and increased cell proliferation[4]. However, it is not yet clear how Beclin 1 modulates cell death in cancer cells.

Several studies have reported a role of Beclin 1 in autophagic cell death. When embryonic fibroblasts from Bax/Bak double knockout mice were treated with etoposide, cells underwent a non-apoptotic cell death characterized by the presence of a number of autophagosomes/autolysosomes and the accumulation of both Beclin 1 and the APG5-APG12 complex[5]. Cell death was largely prevented by autophagy inhibitors as well as by silencing of Beclin 1 or APG5. In another study, treatment of mouse L929 fibroblastic cells or human U937 monocytoid cells with a pan-caspase inhibitor induced autophagic cell death[6]. Silencing of Beclin 1 or ATG7, another protein required for the formation of autophagic vacuoles, reduced z-VAD-induced cell death. Beclin 1 physically interacts with the anti-apoptotic proteins, Bcl-2 and Bcl-X_L[7], but the functional significance of this interaction on apoptosis is yet unclear. Thus, although beclin $1^{-/-}$ mouse embryos exhibited widespread cell death and died *in utero*, the *in vitro* apoptotic rate of beclin $1^{-/-}$ embryonic stem cells after UV irradiation or serum withdrawal was not significantly increased compared to wild-type cells[3]. By contrast, mice infected with a recombinant chimera of the neurotropic Sindbis virus combined with full-length human Beclin 1 exhibited

increased survival, fewer apoptotic brain cells and lower brain viral titers than mice infected with chimeras encoding a Beclin 1 protein lacking the Bcl-2 binding domain or with a premature stop codon[7]. However, these results did not provide information on whether the protective effects of Beclin 1 were a consequence of anti-apoptotic effects, anti-viral effects, or a combination of both mechanisms. Lastly, a recent study reported that the over-expression of Beclin 1 in MKN28 human gastric cancer cells stimulated *cis*-diamminedichloroplatinum (CDD)- or doxorubicin-induced cell death, while a Beclin 1 siRNA protected the cells from apoptosis in the same experimental conditions[8].

We have previously observed a significant correlation between Beclin 1 and Bcl-X_L mRNA expression in human hepatocarcinoma (unpublished results), suggesting that Beclin 1 over-expression may prevent hepatocyte apoptosis. In order to test this assumption, we investigated the effect of Beclin 1 silencing on the response of HepG2 cells to apoptotic stimuli. In the present study, we showed that partial Beclin 1 silencing aggravates the mitochondrial permeabilization and the apoptotic response induced by an agonistic anti-Fas antibody or by doxorubicin in a human HCC cell line.

MATERIALS AND METHODS

Materials

HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mL/L fetal calf serum and 1% penicillin/streptomycin sulfate (Gibco, Invitrogen).

Small interfering RNA (siRNA) duplexes were purchased from Dharmacon (Lafayette, CO). We used the beclin 1 siRNA: 5'-CAGUUACAGAUGGAGCUAA tt-3' (nt 655-673), which is located downstream from the initiation codon of the human beclin coding region, and a luciferase siRNA as a control siRNA: 5'-CUUACGCU GAGUACUUCGAtt3'. HepG2 cells (15-25 × 10^6 cells) were collected by trypsinization. The pellet was washed with serum-free OptiMEM (Gibco, Invitrogen) without antibiotics, and cells were resuspended in 500-600 μL of the same medium containing 12 μmol/L siRNA. After 10 min on ice, cells were electroporated at 230 V, 960 μF. Cells were resuspended in DMEM containing 100 mL/L fetal calf serum without antibiotics, and were plated in 60 mm Petri dishes (approximately 10^6 cells per dish). Antibiotics were added 3 h later.

In a first treatment protocol, 5 μg/mL of an agonistic anti-Fas antibody (7C11, Immunotech, Beckman Coulter, Miami, FL) was added 24 h after siRNA transfection, with or without 10 μmol/L benzyloxycarbonyl-valyl-alanyl-aspartic acid (o-methyl)-fluoro-methylketone (z-VAD-fmk) (Alexis, Coger, Paris, France), a pan-caspase inhibitor, and apoptosis was detected 40 h later, except for the assessment of cytochrome *c* release, which was detected at 24 h.

In a second treatment protocol, 6 μmol/L doxorubicin (Sigma, Saint-Louis, MO) was added 48 h after siRNA transfection, with or without 100 μmol/L z-VAD-fmk, and apoptosis was detected 24 h later except for the assessment of cytochrome c release, which was detected at 20 h.

Measurement of Beclin 1 mRNA expression by ribonuclease protection assay (RPA)

Attached HepG2 cells were washed twice with PBS and lysed in 5 mol/L guanidine thiocyanate and 0.1 mol/L EDTA (pH 7.4). RPA was carried out on cell lysates without previous RNA extraction[9]. The following DNA fragments were used to prepare riboprobes: a 200-bp fragment (nt 554-753, accession no. AF139131) from human beclin 1, and a 131-bp fragment (nt 553-683, accession no. NM00101) from human S6 used to normalize mRNA levels. The RPA and the separation of the protected mRNA fragments by polyacrylamide gel electrophoresis were performed as previously described[9]. Gel radioactivity was assessed with an Instant Imager (PerkinElmer, Courtaboeuf, France), and the ratio of the studied mRNA to the S6 mRNA was computed.

Western blot analysis of Beclin 1 and Bcl-X_L

Attached HepG2 cells were washed twice with PBS, harvested in PBS, and pelleted by centrifugation. The pellet was weighed and suspended in 2 × Laemmli buffer. Proteins were separated on SDS-polyacrylamide gels (70 g/L polyacrylamide for Beclin 1 and 120 g/L for Bcl-X_L) and transferred onto nitrocellulose membranes. After blocking, membranes were incubated for 1 h with either the anti-Beclin 1 antibody supplied by Dr Yoshimori[10], or an anti-Bcl-$X_{S/L}$ antibody (S-18) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Immunotech, Beckman Coulter). Specific bands were detected by an enhanced chemiluminescence system (Pierce, Rockford, IL). Membranes were subsequently probed with a monoclonal anti-β-actin antibody (Sigma), and the β-actin signal was used to normalize protein loading.

Poly(ADP-ribose) polymerase (PARP) cleavage

Attached and floating HepG2 cells were harvested in culture medium and pelleted by centrifugation. Nuclear proteins were extracted according to Sadowski and Gilman[11]. After protein measurement (Biorad Reagent, Pierce), nuclear extracts underwent SDS-7% polyacrylamide gel electrophoresis and blotting as described above, and were revealed with a monoclonal anti-human PARP antibody (clone C2-10, BD Pharmingen, San Diego, CA).

Assessment of cell DNA content by flow cytometry

Pooled, free and adherent HepG2 cells were centrifuged, washed with PBS and fixed with 700 mL/L cold ethanol. The pellet was resuspended in 100 μL RNAse A (180 μg/mL) and incubated at room temperature for 30 min. Propidium iodide (Merck, Darmstadt, Germany; final concentration, 50 μg/mL) was added, and cells were incubated at room temperature in the dark for 15 min. The percentage of cells with a low DNA content (subG1 cells) was quantified by flow cytometry using an Epics Elite ESP coulter cytometer (Beckman coulter, Fullerton, CA). Propidium iodide was excited at 488 nm and fluorescence was analyzed at 630 nm.

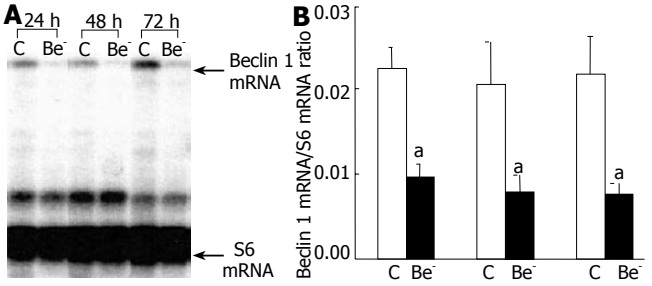


Figure 1 Beclin 1 mRNA expression after transfection of HepG2 cells with a Beclin 1 siRNA or a control siRNA. **A:** Representative RPA gel; **B:** Quantification of the Beclin1/S6 mRNA ratio. Results are means ± SE for 4 experiments ($^aP < 0.05$ *vs* C).

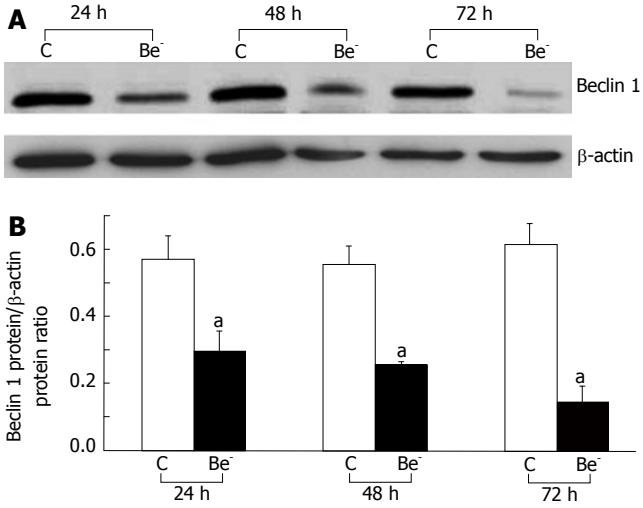


Figure 2 Beclin 1 protein expression after transfection of HepG2 cells with a Beclin 1 siRNA or a control siRNA. **A:** Representative Western blot of Beclin 1 and β-actin; **B:** Quantification of the Beclin 1 protein normalized to β-actin. Data are means ± SE for three experiments ($^aP < 0.05$ *vs* C).

Assessment of the mitochondrial membrane potential by flow cytometry

To determine the mitochondrial membrane potential, we used DIOC6(3) (Molecular Probes, Eugene, OR), a lipophilic cationic dye, which accumulates into polarized mitochondria. Free and adherent HepG2 cells were collected, rinsed with PBS, and suspended in the culture medium. DIOC6(3) was added at a final concentration of 100 nmol/L, and cell suspensions were incubated at 37 ℃ in the dark for 30 min. Propidium iodide (1.5 μmol/L) was added before flow cytometry to exclude dead cells and to only take into account the fluorescence DIOC6(3) in living cells. DIOC6(3) fluorescence was measured by flow cytometry. DIOC6(3) was excited at 488 nm and detected at 510 nm.

Measurement of cytochrome c release

Mitochondrial and cytosolic extracts were prepared using the ApoAlert® cell fractionation kit (BD Biosciences Clontech, Mountain View, CA, USA). Equal levels of protein were separated on SDS-12.5% polyacrylamide gel electrophoresis, transferred and immunoblotted with an anti-cytochrome *c* antibody supplied with the kit.

Statistical analysis

Data were expressed as means ± SE. An unpaired Student's *t* test was used to assess statistical differences between HepG2 cells transfected with the control siRNA or the Beclin 1 siRNA. A paired Student's *t* test was used to assess differences resulting from treatments in control siRNA- or in Beclin 1 siRNA-transfected HepG2 cells.

RESULTS

Effects of partial Beclin 1 silencing on Fas- and doxorubicin-mediated apoptosis

We used RNA interference to partially silence the Beclin 1 gene in HepG2 cells. The Beclin 1 mRNA was decreased by 57%, 61% and 65%, 24, 48 and 72 h after transfection of the Beclin 1 siRNA, respectively (Figure 1), and the Beclin 1 protein was decreased by 48%, 54% and 76%, respectively (Figure 2).

We then measured the percentage of cells with a decreased DNA content (subG1 cells) as a consequence of DNA fragmentation after treatment of transfected cells with doxorubicin or with an agonistic anti-Fas antibody, which both trigger apoptotic cell death in HepG2 cells[12,13]. We compared the percentage of cells with a subG1 DNA content in HepG2 cells transfected with the control siRNA (control cells) or the Beclin 1 siRNA (Be⁻ cells) (Figure 3). The percentage of subG1 cells was not significantly increased in the untreated Be⁻ cells compared to the untreated control cells (Figure 3B). However, after exposure to the anti-Fas antibody or doxorubicin, the increase in subG1 cells was much higher in the Be⁻ cells compared to the control cells (Figure 3B). In both control cells and Be⁻ cells, the pan-caspase inhibitor z-VAD-fmk markedly decreased subG1 cells after treatment with the anti-Fas antibody or doxorubicin (Figure 3C).

We also studied the apoptotic response of HepG2 cells by assessing PARP cleavage (Figure 4). The cleaved p85 fragment of PARP was not detected in the untreated control cells or untreated Be⁻ cells. After treatment with the anti-Fas antibody, the cleaved p85 fragment was still absent in the treated control cells, but present in the treated Be⁻ cells. After treatment with doxorubicin, the p85 fragment was more abundant, and the uncleaved p116 fragment less abundant, in the Be⁻ cells compared to the control cells (Figure 4).

Since the apoptotic effects of Fas or doxorubicin are amplified by mitochondria[14-16], we determined whether Beclin 1 depletion increases the mitochondrial pathway of apoptosis by first assessing changes in the mitochondrial membrane potential (Figure 5). The percentage of living cells with a low mitochondrial membrane potential was not significantly increased in the untreated Be⁻ cells compared to the untreated control cells. After treatment with the anti-Fas antibody or doxorubicin, the percentage of living cells with a low mitochondrial membrane potential was significantly higher in the Be⁻ cells than that in the control cells (Figure 5). We next investigated whether Beclin 1 silencing increases the release of mitochondrial cytochrome *c* into the cytosol after treatment with the anti-Fas antibody or doxorubicin[14,16]. We firstly checked that the cytosolic fraction was not contaminated by

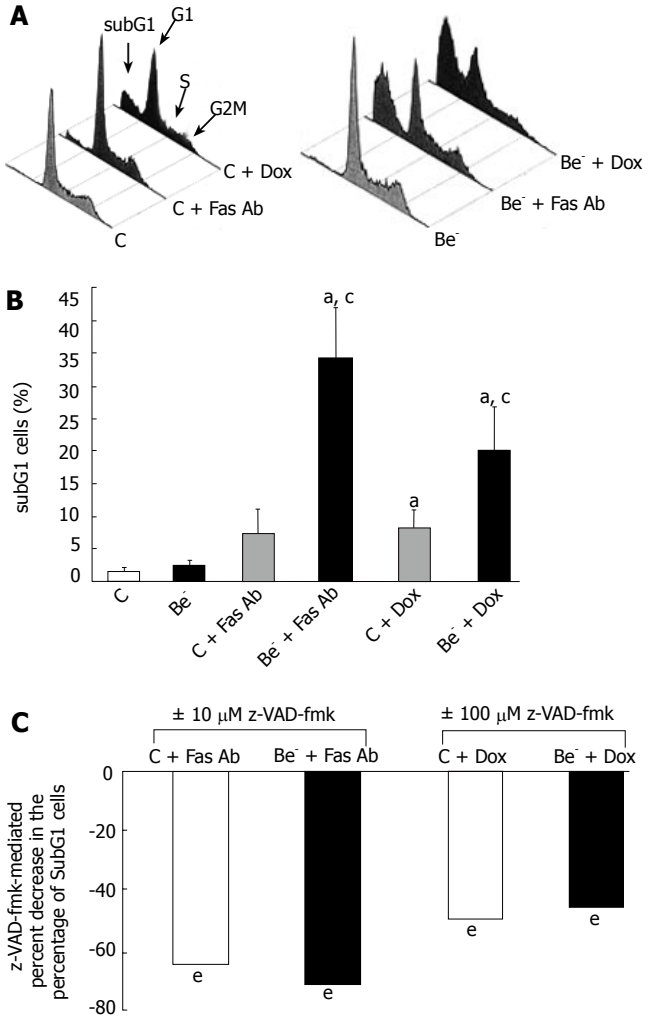


Figure 3 Percentage of subG1 cells after treatment of siRNA-transfected HepG2 cells with an agonistic anti-Fas antibody or doxorubicin. **A:** Representative flow cytometry analysis of the cell cycle; **B:** Quantification of the subG1 peak. Results are means ± SE for 5 to 8 experiments ($^{a}P < 0.05$ *vs* untreated cells; $^{c}P < 0.05$ *vs* cells transfected with the control siRNA); **C:** Effects of z-VAD-fmk. The figure shows the mean percent decrease in subG1 cells for 3 to 5 experiments ($^{e}P < 0.05$ between cells incubated with and without z-VAD-fmk).

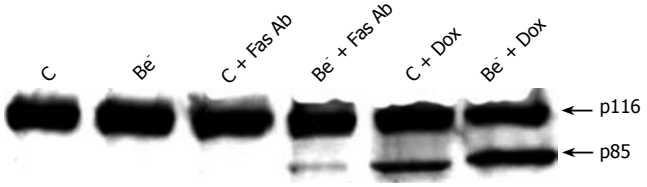


Figure 4 Western blot analysis of PARP cleavage after treatment of siRNA-transfected HepG2 cells with an agonistic anti-Fas antibody or doxorubicin.

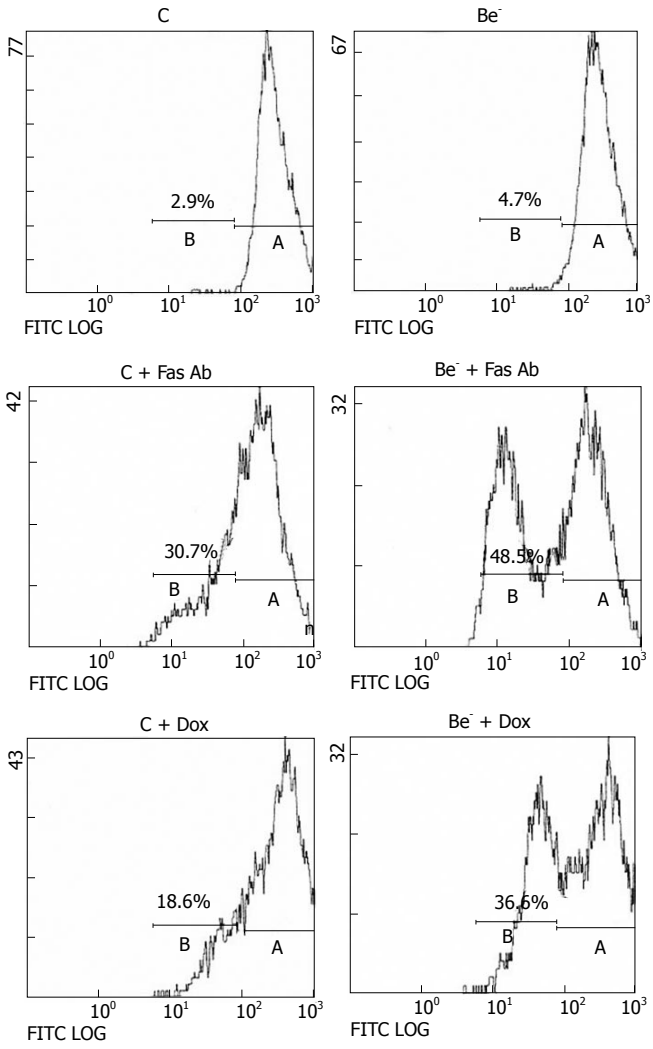


Figure 5 Assessment of the mitochondrial membrane potential in HepG2 living cells after siRNA transfection and treatment with an agonistic anti-Fas antibody or doxorubicin (Dox). In four to five different experiments, the percentage (mean ± SE) of cells with a low mitochondrial membrane potential was 4.5 ± 1.3% in C cells, 7.7 ± 2.9% in the Be- cells, 29.1 ± 4.9% in C cells treated with the Fas Ab, 52.5 ± 7.1% in Be- cells treated with the Fas Ab ($P < 0.05$ *vs* treated C cells), 23.6 ± 3% in C cells treated with Dox, and 38.8 ± 3.8% in Be- cells treated with Dox ($P < 0.05$ *vs* treated C cells).

mitochondria by assessing COX4 (data not shown). Cytosolic cytochrome *c* was increased by the anti-Fas antibody or the doxorubicin treatment in the control cells, and this increase was further enhanced in the Be- cells compared to the control cells, particularly after treatment with the anti-Fas antibody (Figure 6).

To test whether this effect could be mediated by decreased Bcl-X_L expression, we assessed this protein in the untreated and treated cells (Figure 7). Bcl-X_L was not significantly decreased 24, 48 or 72 h post-transfection in the cells transfected with Beclin 1 siRNA compared to the cells transfected with the control siRNA (Figures 7A-B). Treatment with the anti-Fas antibody caused no further changes (Figures 7C-D). Although treatment with doxorubicin tended to decrease Bcl-X_L expression in the control cells, and even more in the Be- cells, the doxorubicin-mediated decline in Bcl-X_L did not differ significantly between the control cells and Be- cells (Figures 7C -D).

DISCUSSION

Although beclin 1 haploinsufficiency is involved in several extrahepatic human cancers[1,4], and increases the frequency of various tumors, including HCC in transgenic mice[2,3], its role in human HCC is still not clear[17], and its potential involvement in liver apoptosis has not been investigated

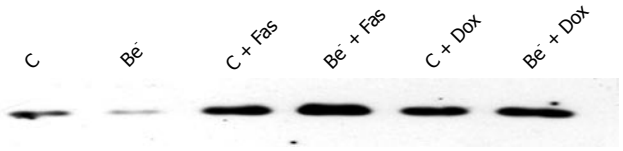


Figure 6 Assessment of cytochrome c release in HepG2 cells after siRNA transfection and treatment with an agonistic anti-Fas antibody or doxorubicin.

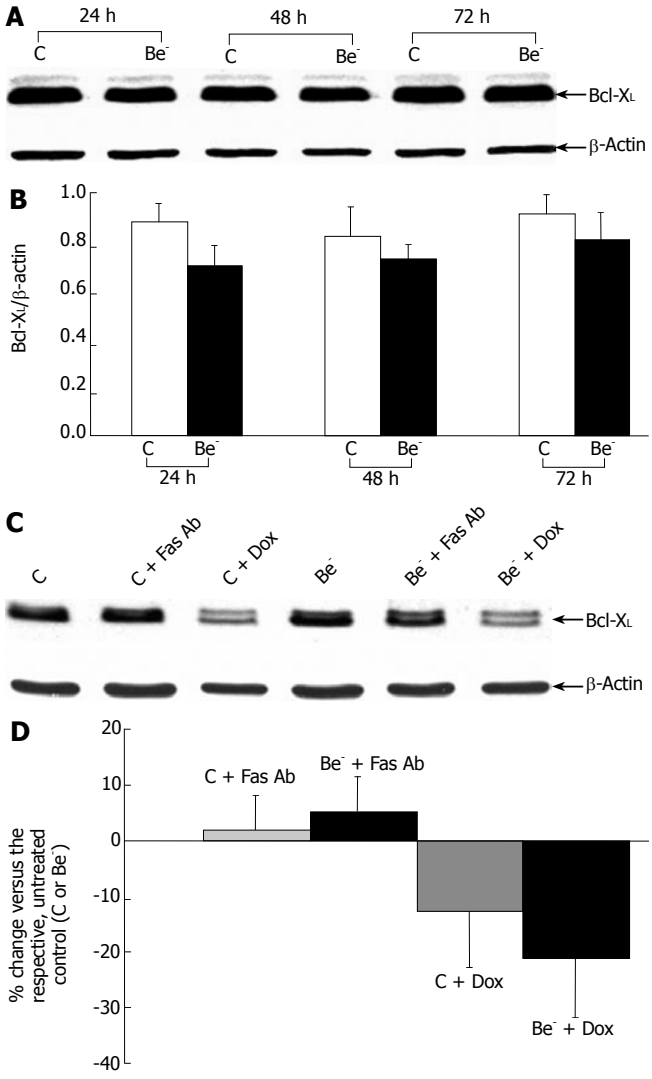


Figure 7 Expression of the Bcl-XL protein after siRNA transfection, and treatment with an agonistic anti-Fas antibody or doxorubicin (Dox). **A:** Representative Western blot of Bcl-XL and β-actin; **B:** Quantification of the Bcl-XL/β-actin ratio (mean ± SE for 5 experiments); **C:** Representative Western blot of Bcl-XL and β-actin after incubation with or without Dox or an anti-Fas; **D:** Quantification of the Fas Ab- or Dox-induced change in Bcl-XL expression (means ± SE for 4 to 5 experiments).

yet. The aim of the present study was to assess the effects of partial Beclin 1 silencing on the susceptibility of HepG2 human hepatoma cells to undergo apoptosis.

In the cells transfected with a Beclin 1 siRNA (Be- cells), the Beclin 1 protein was decreased by 50% at 24 h and by 75% at 72 h compared to the cells transfected with a control siRNA (control cells). Although the spontaneous apoptotic rate of the Be- cells was not significantly increased over that of the control cells, their apoptotic response to doxorubicin or an agonistic anti-Fas antibody was markedly aggravated (Figure 3). The percentage of subG1 cells was much higher in the treated Be- cells compared to the treated control cells (Figures 3A-B) and dropped significantly when cells were pre-treated with the pan-caspase inhibitor, z-VAD-fmk (Figure 3C). In addition, PARP cleavage was more extensive in the treated Be- cells than that in the treated control cells, especially after doxorubicin poisoning (Figure 4).

Two reasons prompted us to investigate the potential effect of Beclin 1 depletion on the mitochondrial pathway. Firstly, Beclin 1 physically interacts with Bcl-2 or Bcl-XL[7,8], two anti-apoptotic proteins predominantly localized to the mitochondrial membrane that prevent loss of mitochondrial membrane potential and cytochrome *c* release[18-20]. Secondly, the apoptotic effects of Fas or doxorubicin are amplified by mitochondria, in particular in hepatocytes and hepatoma cell lines[14-16]. The dissipation of mitochondrial membrane potential after anti-Fas antibody or doxorubicin treatments was more severe, and the cytochrome *c* release was more abundant in the Be- cells than that in the control cells (Figures 5 and 6), suggesting that Beclin 1 depletion activates the mitochondrial pathway of apoptosis. In a previous study, the over-expression of full-length human Beclin 1 was associated with decreased neuronal apoptosis in mice infected with the neurotropic Sinbis virus, whereas a deleted Beclin 1 construct lacking the region interacting with Bcl-2 or Bcl-XL was not protective[7]. This previous observation may suggest that the interaction of Beclin 1 with either Bcl-2 or Bcl-XL is essential for the anti-apoptotic effects of Beclin 1[7]. Since several studies have reported that Bcl-2 is not constitutively expressed in HepG2 cells[21-23] and Bcl-2 over-expression does not protect these cells from Fas or doxorubicin treatment[22-24], Bcl-XL seems to be the major anti-apoptotic protein in the HepG2 cell line. In the present study, partial Beclin 1 silencing increased apoptosis without significantly decreasing the Bcl-XL protein (Figure 7), suggesting that normal Beclin 1 levels are required to protect HepG2 cells from apoptotic stimuli, possibly by maintaining the expression of the Bcl-XL-Beclin 1 complex. How does the interaction between Beclin 1 and Bcl-XL or other proteins act to modulate mitochondrial permeabilization and apoptosis remains to be determined.

In contrast to our results, Furuya *et al*[8] instead observed that Beclin 1 over-expression aggravated CDD- or doxorubicin-induced apoptosis in a gastric cancer cell line, while Beclin 1 silencing had the opposite effects[8]. However, Beclin 1 silencing did not protect another gastric cancer cell line (with lower Bcl-2 and Bcl-XL expression) against CDD-mediated apoptosis. It thus appears that Beclin 1 differently modulates apoptosis in different cell lines. Although reasons for these differences are unknown, changes in the molecular environment of Beclin 1 from one cancer cell line to another might be involved, including different expression of Bcl-2 and Bcl-XL[8], and other pro-apoptotic proteins of the Bcl-2 family interacting with these proteins, but not with Beclin 1[7,8,24,25]

In conclusion, our data demonstrate that partial Beclin 1 silencing augments the mitochondrial permeabilization and the apoptosis induced by Fas stimulation or doxorubicin treatment in hepatoma-derived HepG2 cells. As Takehara *et al*[21] reported that down-expression of Bcl-XL by antisense oligonucleotide aggravates apoptosis induced by staurosporine or serum deprivation in HepG2 cells, the use of both Beclin 1 and Bcl-XL antisense oligonucleotides in combination with chemotherapeutic drugs might efficiently trigger apoptosis in human hepatocarcinoma. The different effects of Beclin 1 on apoptosis in different cell lines[3,7,8] show that Beclin 1, unlike Bcl-2 or Bcl-XL, can be pro-apoptotic, anti-apoptotic or non-effective, depending on the cellular context.

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BASIC RESEARCH

Down-regulation of survivin expression by small interfering RNA induces pancreatic cancer cell apoptosis and enhances its radiosensitivity

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Abstract

AIM: To investigate the inhibitory effect of small interfering RNA (siRNA) on the expression of survivin in pancreatic cancer cell line PC-2 and the role of siRNA in inducing PC-2 cell apoptosis and enhancing its radiosensitivity.

METHODS: A siRNA plasmid expression vector against survivin was constructed and transfected into PC-2 cells with Lipofectamine™ 2000. The down regulation of survivin expression was detected by semi-quantitive RT-PCR and immunohistochemical SP method and the role of siRNA in inducing PC-2 cell apoptosis and enhancing its radiosensitivity was detected by flow cytometry.

RESULTS: The sequence-specific siRNA efficiently and specifically down-regulated the expression of survivin at both mRNA and protein levels. The expression inhibition ratio was 81.25% at mRNA level detected by semi-quantitive RT-PCR and 74.24% at protein level detected by immunohistochemical method. Forty-eight hours after transfection,apoptosis was induced in 7.03% cells by siRNA and in 14.58% cells by siRNA combined with radiation.

CONCLUSION: The siRNA plasmid expression vector against survivin can inhibit the expression of survivin in PC-2 cells efficiently and specifically. Inhibiting the expression of survivin can induce apoptosis of PC-2 cells and enhance its radiosensitivity significantly. RNAi against survivin is of potential value in gene therapy of pancreatic cancer.

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Key words: RNA interference; Survivin; Pancreatic cancer; Radiation

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INTRODUCTION

Survivin, a structurally unique member of the inhibitors of apoptosis protein (IAP)family, is involved in the control of cell division and inhibition of apoptosis. Survivin inhibits apoptosis via its baculovirus inhibitor of apoptosis repeat (BIR) protein domain by either directly or indirectly interfering with the function of caspases. Survivin is also a chromosomal passenger protein that is required for cell division. Survivin is expressed in embryonic tissues as well as in the majority of human cancers, but not in most normal adult tissues. The cancer-specific expression of survivin, coupled with its importance in inhibiting cell apoptosis and regulating cell division, makes it a useful diagnostic marker and a potential target for cancer treatment[1].

RNA interference (RNAi) is a process of post transcriptional gene silencing (PTGS) in which double-stranded RNA (dsRNA) inhibits gene expression in a sequence-dependent manner *via* degradation of the corresponding mRNA [2-5]. RNAi is initiated by an event while dsRNAs are recognized by Dicer, a member of the RNase III protein family[6]. The Dicer enzyme cleaves dsRNAs into 21 to 23 nucleotide short siRNAs. These siRNA duplexes are incorporated into a protein complex called the RNA-induced silencing complex, which recognizes and cleaves the cognate mRNA[7, 8]. Recently, Elbashir and his colleagues reported that introduction of synthetic 21nt siRNA into

mammalian cells could suppress gene expression in a sequence-specific manner[9]. By using dsRNAs of such a small size, the activation of dsRNA-dependent protein kinase and 2', 5'- oligoadenylate synthetase, which occurs as an interferon response to long dsRNA (i.e. longer than 30nt), leading to a global decrease in protein synthesis, is avoided [10,11]. Thus, siRNA has now become a powerful tool for studies on gene function, cancer and viral disease therapy. In this study, a siRNA against survivin plasmid expression vector was constructed and transfected into PC-2 cells. The changes of survivin expression following RNAi and the role of siRNA in inducing PC-2 cell apoptosis and enhancing its radiosensitivity were investigated, which may lay a foundation for further studies on the functions of survivin and genetic therapy involved in human pancreatic cancer.

MATERIALS AND METHODS

Cell line and culture

Human pancreatic cancer cell line PC-2 was purchased from the Medical Experimental Animal Center of the Fourth Military Medical University. The cells were cultured in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum (FBS), 1×10^5 U/L penicillin and 0.1 g/L streptomycin in a humidified incubator containing 50 mL/L CO_2 at 37 ℃.

Construction of siRNA plasmid expression vector against survivin

Plasmid vector Pgenesil-1 was purchased from Wuhan Genesil Biotechnology Co, Ltd. Aiming at the sequence of survivin[12] (5'-GGA CCA CCG CAT CTC TAC A-3'), two DNA chains were synthesized as following: 5'-GATCC GGA CCA CCG CAT CTC TAC A TTCAAGACG TGT AGA GAT GCG GTG GTC C TTTTTT GAATTC A-3' and 3'-G CCT GGT GGC GTA GAG ATG T AAGTTCTGC ACA TCT CTA CGC CAC CAG G AAAAAA CTTAAG TTCGA-5'. The structure of the DNA chains is *Bam*HI + sense chain + loop + antisense chain + termination signal + *Eco*RI + *Hin*dIII. Equal amount of the two DNA chains was annealed and phosphorylated, then the double-stranded DNA was ligated with the linearized plasmid vector Pgenesil-1 completely digested with *Bam*HI and *Hin*dIII. The ligated plasmid DNA was transfected into competent cells $DH5\alpha$ which were then seeded on solid LB medium containing 0.05 g/L kanamycin and cultured at 37 ℃ overnight. Three monoclonal colonies were picked out and seeded in 3 mL LB culture fluid containing 0.05 g/L kanamycin and cultured at 37 ℃ overnight in a rocking bed. The plasmid was extracted and verificated by digesting with restriction endonucleases and sequencing. The new plasmid was named Pgenesil-sur(+). The negative control plasmid Pgenesil-sur(-) was constructed as the same process. The target sequence is 5'-GAC TTC ATA AGG CGC ATG C-3' and does not have any homology with human beings or mice. The sequences of the two DNA chains are 5'-GATCC GAC TTC ATA AGG CGC ATG C TTCAAGACG GCA TGC GCC TTA TGA AGT C TTTTTT GTCGAC A-3' and 3'-G CTG AAG TAT TCC GCG TAC G AAGTTCTGC CGT ACG CGG AAT ACT TCA G AAAAAA CAGCTG TTCGA -5'. Their structure is *Bam*HI + sense chain + loop + antisense chain + termination signal + *Sal*I + *Hin*d III.

Extraction of plasmids

The plasmid extraction kit without endotoxin was purchased from Beijing Tianwei Corporation. The plasmids were extracted following the manufacturer's instructions. The concentration and purity of the plasmids were detected with an ultraviolet spectrophotometer. The plasmids were stored at -20 ℃ for following experiments.

Transfection of plasmids

Transfection reagent Lipofectamine™2000 was purchased from Invitrogen Corporation. Transfection was performed following the manufacturer's instructions. The ratio of plasmids: Lipofectamine™2000 was 1:3. Twenty-four hours before the transfection, the common complete medium was replaced by the antibiotics- free medium containing serum. Six hours after the transfection, the medium was replaced by the common complete medium again. Twelve hours after the transfection, expression of EGFP in PC-2 cell was observed under an inverse fluorescence microscope.

Expression of survivin mRNA detected by semi-quantitive RT-PCR

PC-2 cells were seeded in 6cm culture capsules and divided into blank control group, negative control group and positive experiment group. Each group contained 3 culture capsules. Only Lipofectamine™2000 was used for the transfection in the blank control group, plasmid Pgenesil-sur(-) was used for the transfection in the negative control group and plasmid Pgenesil-sur(+) was used for the transfection in the positive experiment group. Twenty-four hours after the transfection, 1×10^6 cells were collected and total RNA was extracted using the Trizol reagent following the manufacturer's instructions. The concentration and purity of the total RNA were detected with ultraviolet spectrophotometer. RT-PCR was performed by two-step method. Synthesis of cDNA was performed using the RevertAid™ first chain cDNA synthesis kit following the manufacturer's instructions. PCR was performed using the one tube convenient PCR kit purchased from Beijing Tianwei Corporation. Amplification of human GAPDH served as an internal standard. The primers used are 5'-CGA AGT CAA CGG ATT TGG TCG TAT-3' (forward primer) and 5'-AGC CTT CTC GGT GGT GAA GAC-3' (reverse primer). The amplification product was 306bp.The primers of survivin are 5'-GCA TGG GTG CCC CGA CGT TG-3' (forward primer) and 5'-GCT CCG GCC AGA GGC CTC AA -3' (reverse primer). The amplification product was 447bp. The PCR products were separated in 1.5% agarose gels, visualized by staining with ethidium bromide. Semi-quantitative analysis was performed with the Dolphin 1D software. The expression intensity of survivin was denoted with the ratio of the photodensity of the RT-PCR products of survivin and GAPDH. The inhibition ratio of survivin expression was calculated with the following formula: inhibition ratio of survivin expres-

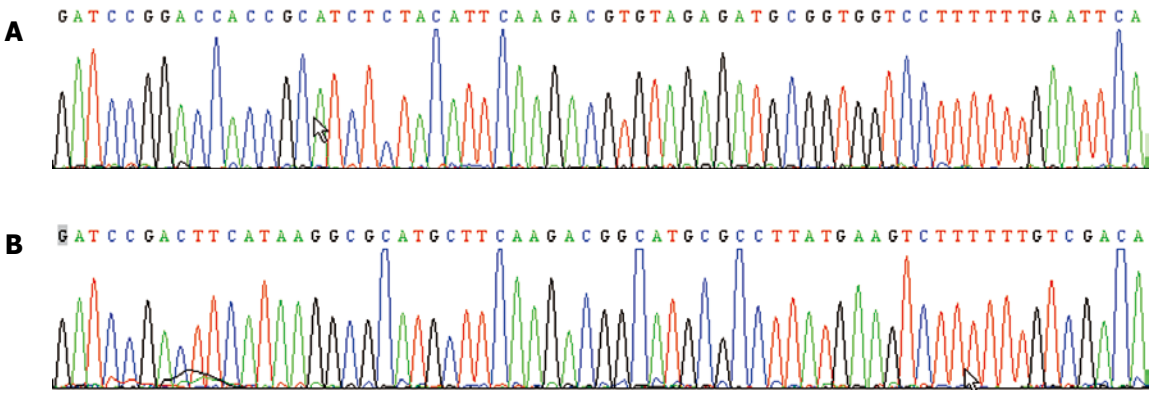


Figure 1 Reports of the sequencing of plasmid Pgenesil-sur(+) (**A**) and plasmid Pgenesil-sur (-) (**B**).

sion = (1-the expression intensity of survivin in the observation group/ the expression intensity of survivin in the blank control group)×100%.

Expression of survivin protein detected by immunohistochemical method

PC-2 cells were seeded in 24-well plates with a small square cover slip in each well. Grouping and transfection were performed the same as above. Each group contained 8 wells. Twenty-four hours after the transfection, the cells were fixed with cold 65% liquor pyroaceticus. The expression of survivin protein was detected by immunohistochemical SP method. The anti-survivin antibody was a rabbit anti-human monoclonal antibody purchased from Beijing Zhongshan Corporation. The working concentration was 1:50. A petroline section of breast cancer expressing survivn positively was used as a positive control, PBS was used to replace the anti-survivn antibody to make a negative control. Cells with brown-yellow granulations in endochylema or nuclei were considered as positive cells. According to the coloration, the cells were scored as following: 0, no staining; 1, faint-yellow; 2, brown-yellow; 3, dark-brown. Five fields of vision of each cover slip were observed under high power lens randomly. The cells were calculated and scored. The sum of the scores was divided by the number of cells, the result was considered as the expression intensity of survivin. The inhibition ratio of survivin expression was calculated with the following formula: inhibition ratio of survivin expression = (1-the expression intensity of survivin in the observation group/ the expression intensity of survivin in the blank control group)× 100%.

Radiation and apoptosis of PC-2 cells detected by flow cytometry

Cells were seeded in 25 cm^2 culture flasks and divided into blank control group, negative control group, positive experiment group, blank control + radiation group, negative control + radiation group, and positive experiment + radiation group. Each group contained 5 culture flasks. Transfection was performed the same as above. Twenty-four hours after the transfection, cells of groups 4, 5, 6 were radiated with 15 Gy of X-ray(6MV) at room temperature using a Varian 2300 C/D linear accelerator. The radiation conditions were ounce-skin distance = 100 cm, radiation dose rate=2 Gy/min. After radiation, the cells were continuously cultured for another 24 h, digested, collected and washed twice with PBS. Then the cells were fixed with cold 75% ethanol at 4 ℃ overnight. The cells were centrifuged at 1000 r/ min for 5 min, the supernate was poured out, the cells were washed twice with PBS. The cells were resuspended with PBS containing 0.05 g/L RNase and incubated for 30 min at room temperature avoiding light to eliminate the intracellular RNA. The cells were centrifuged at 1000 r/min for 5 min, the supernate was poured out. The cells were resuspended with PBS containing 0.06 g/L propidium iodode again and incubated for 30 min at room temperature avoiding light, then cell apoptosis was detected by flow cytometry. The management was the same in cells of groups 1-3 except for no radiation.

Statistical analysis

Data were expressed as mean ± SD. All statistical analyses were performed using SPSS 10.0 software(one-way ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Verification of plasmids

The multiclone sites of plasmid Pgenesil-1 were as following: *Hin*dIII-*Xba*I-*Sal*I -*Pst*I-*Bam*HI-U6 Promotor-*Eco*RI-*Sal*I-*Xba*I-*Dra*III. An *Eco*RI site for plasmid Pgenesil-sur(+) and a *Sal*I site for plasmid Pgenesil-sur(-) were designed in the inserted fragments between the sites of *Bam*HI and *Hin*dIII. If the insertion was correct, a band about 400 bp should be cut off by *Eco*RI or *Sal*I. The results of digestion with restriction endonucleases and sequencing showed correct plasmids(Figure 1).

Results of transfection

Twelve hours after plasmid Pgenesil-sur(+) or plasmid Pgenesil-sur(-) was transfected into PC-2 cells using Lipofectamine^{TM}2000, expression of EGFP could be observed under an inverse fluorescence microscope. Twenty-four hours after the transfection, the expression of EGFP was the strongest. Seventy-two hours after the transfection, the expression of EGFP was attenuated gradually(Figure 2).

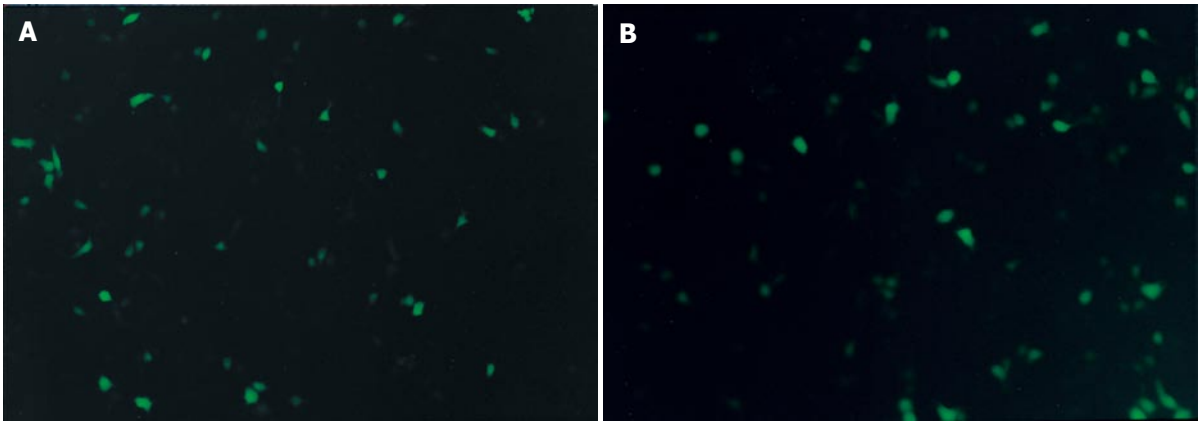


Figure 2 Expression of EGFP in PC-2 cells 24 h after trancfection of plasmid Pgenesil-sur(+) (**A**) and plasmid Pgenesil-sur(-) (**B**) (original magnification×200).

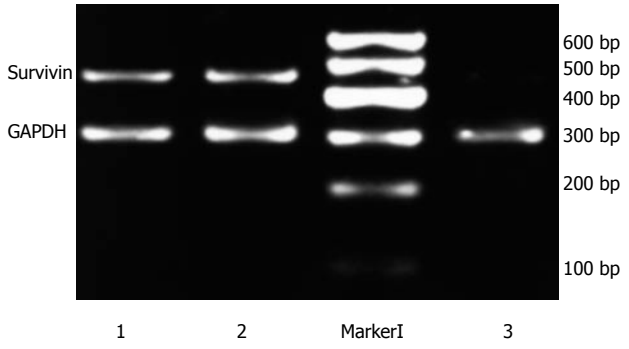


Figure 3 Down-regulation of survivin mRNA expression detected by semi-quantitive RT-PCR. 1: The blank control group; 2: The negative control group; M: Marker I; 3: The positive experiment group.

Significant down regulation of survivin mRNA expression by siRNA

Twenty-four hours after the transfection, the expression intensities of survivin mRNA in blank control, negative control and positive experiment groups were 0.96±0.02, 0.98 ± 0.03 and 0.18 ± 0.03, respectively. The statistical analysis showed that the expression of survivin mRNA in PC-2 cells was down regulated significantly after transfection with plasmid Pgenesil-sur(+) ($P < 0.05$), the inhibition ratio was 81.25%. Then expression of PC-2 cells transfected with plasmid Pgenesil-sur(-) had no effect on the expression of survivin mRNA ($P > 0.05$)(Figure 3).

Significant down regulation of Survivin protein expression by siRNA

Twenty-four hours after the transfection, the expression intensities of survivin protein in blank control, negative control and positive experiment groups were 2.29 ± 0.23, 2.34 ± 0.15 and 0.59 ± 0.16, respectively. The statistical analysis showed that the expression of survivin protein in PC-2 cells was down- regulated significantly after transfected with plasmid Pgenesil-sur(+) ($P < 0.05$), the inhibition ratio was 74.24%. The expression of PC-2 cells transfected with plasmid Pgenesil-sur(-) had no effect on the expression of survivin protein ($P > 0.05$) (Figure 4).

Role of siRNA in inducing PC-2 cell apoptosis and enhancing its radiosensitivity detected by flow cytometry

Forty-eight hours after transfection, no cell apoptosis was detected in blank control and negative control groups, but in (7.03 ± 1.12)% cells in the positive experiment group. The apoptosis rate in blank control + radiation group, negative control + radiation group and positive experiment + radiation group was (1.77 ± 0.30)%, (1.66 ± 0.46)% and (14.58 ± 2.72)%, respectively. The statistical analysis showed that transfection with plasmid Pgenesil-sur(+) could induce spontaneous apoptosis of PC-2 cells in a certain degree and the effect was better than that of 15 Gy of X-ray radiation. Inhibiting the expression of survivin could enhance the radiosensitivity of PC-2 cells significantly. siRNA against survivin combined with radiation could induce significant apoptosis of PC-2 cells (Figure 5).

DISCUSSION

Pancreatic cancer is one of the most malignant tumors with a very poor prognosis. The 5-year survival rate of pancreatic cancer patients receiving surgery and chemotherapy ranges 1%-2%. One of the reasons for this low survival rate is the insensitivity of pancreatic cancer to most oncologic therapies such as chemotherapy, radiotherapy and immunotherapy. Tumor development and progression as well as resistance to most oncologic therapies result mainly from lacking response to apoptotic stimuli[13].

Survivin, a member of the IAP family, is a bifunctional protein that suppresses apoptosis and regulates cell division. Recent studies showed that there is a close relationship between survivin and malignant tumors. The expression of survivin is highly cancer-specific, and is one of the top four transcripts uniformly up-regulated in human cancers, but not in normal tissues[14]. The overexpression of survivin appears to correlate with aggressive tumor behavior and poor prognosis in colorectal cancer[15-17], neuroblastomas[18], melanomas[19], non small cell lung cancers[20], breast carcinomas[21], esophageal squamous cell carcinomas[22] and soft-tissue

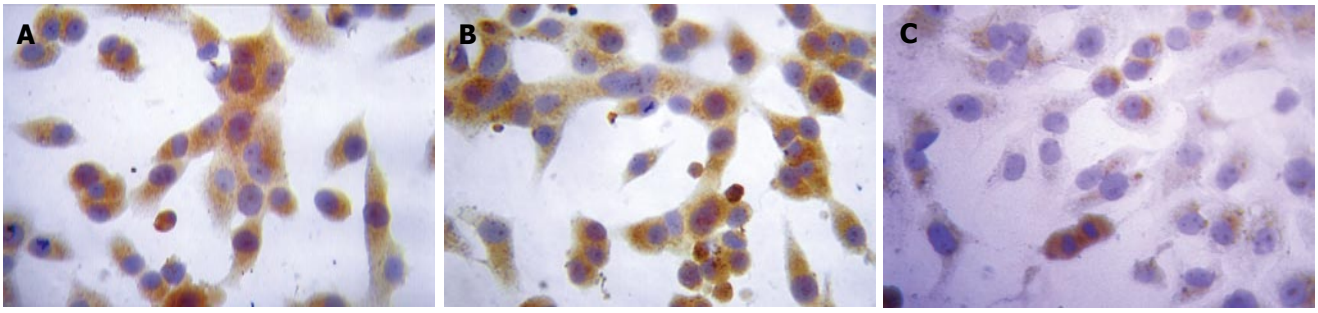


Figure 4 Down-regulation of survivin expression detected by immunohisto- chemical SP method in the blank control group (**A**), negative control group (**B**), and positive experiment group (**C**) (coloration with DAB, original magnification × 400)

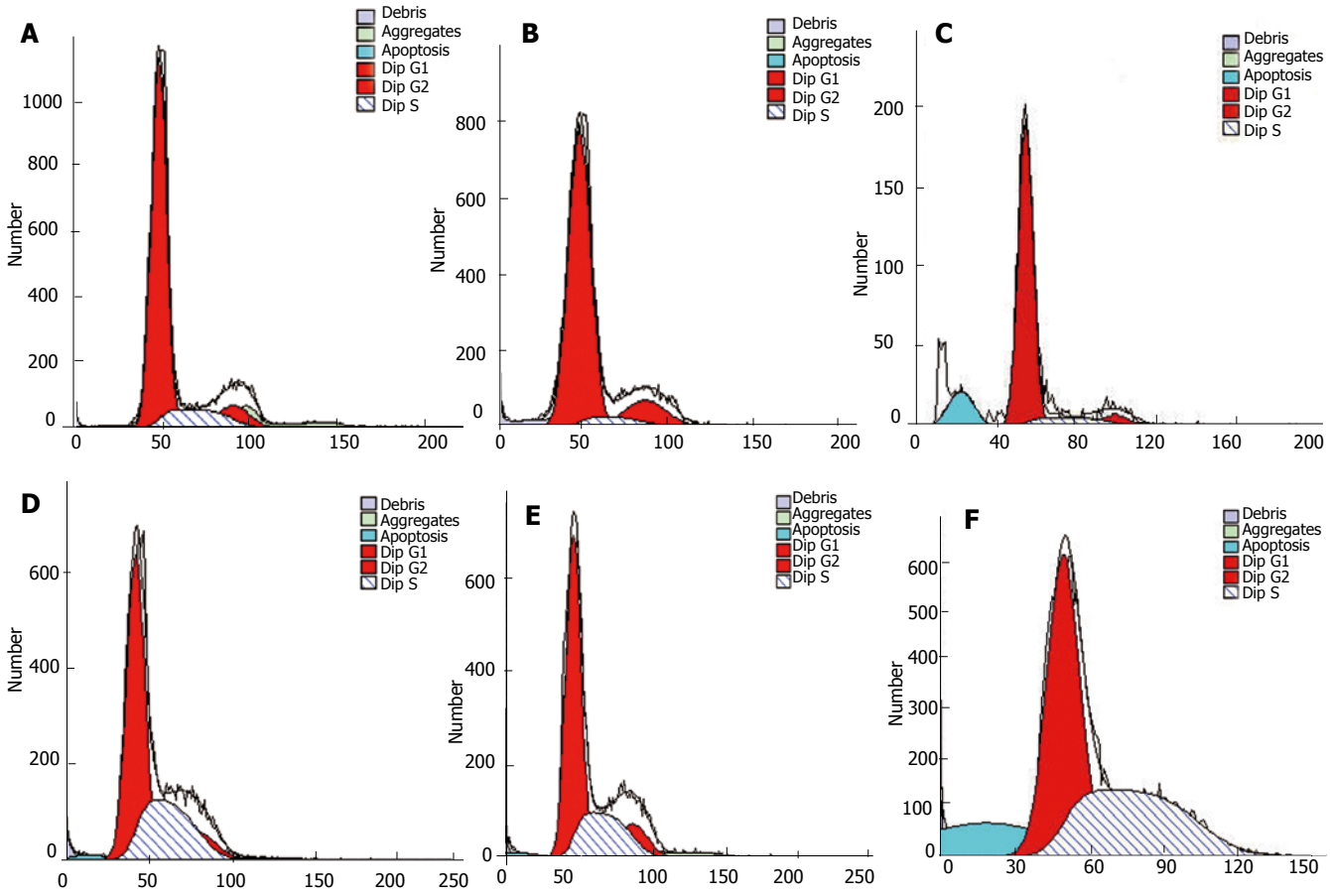


Figure 5 Apoptosis of PC-2 cells detected in 6 groups by flow cytometry (stained with PI)

sarcoma[23]. Additionally, survivin overexpression is also correlated with insensitivity to chemotherapy and radiotherapy in cancers[24,25]. Inhibiting the expression of survivin can induce tumor cell apoptosis, sensitize tumor cells to chemotherapy, radiotherapy and inhibit tumor-angiogenesis[26-29]. Survivin has become an ideal target for the diagnosis and treatment of cancer.

Studies on the relationship between the expression of survivin and pancreatic cancer showed that survivin is also overexpressed in pancreatic cancer, and participates in the development and progress of pancreatic cancer by inhibiting apoptosis. Its overexpression is associated with the insensitivity of pancreatic cancer to chemotherapy and radiotherapy, and is a poor prognosis marker[30-33]. All these studies suggest that survivin is of great potential value in the treatment of pancreatic cancer.

The mechanisms by which survivin inhibits cell apoptosis and cell division are still highly controversial. Survivin may regulate apoptosis by directly or indirectly inhibiting the activity of caspases (caspase-3, -7, -6, -8, -9, -10), or by interacting with cdk4 which releases P_{21} from its complex with cdk4 making it possible for P_{21} to complex with caspase-3, the initial step in inactivating caspase-3 in mitochondria. Survivin may possibly function to inhibit the activity of caspases indirectly *via* binding to and sequestering Smac/DIABLO, thus preventing Smac/DIABLO binding to other IAPs. Survivin might be an additional chromosomal passenger protein just like inner

centromere protein (INCENP), TD-60 and Aurora-B, and is important for cytokinesis and chromosome movement during cell division. Overexpression of survivin may overcome the G2/M phase checkpoint to enforce progression of cells through mitosis[1].

RNAi is characterized by high efficiency, high specificity and low toxicity. siRNA has become a powerful tool for studies on gene function, cancer and viral disease therapy. In this study, we designed and constructed the siRNA plasmid expression vector. Its transcript can form a short hairpin RNA(shRNA) with inverted repeat sequence separated by a short loop sequence, then the shRNA is processed into functional siRNA to degradate target mRNA and silences its expression. The siRNA plasmid expression vector constructed in this study contains the EGFP gene that makes it convenient to observe the result of transfection. By aiming at different sequence of the target gene, the effect of RNAi may be greatly different [34, 35] suggesting that it is very important to find the proper sequence. The sequence of Kappler consulted in this study has been confirmed effective in multiple tumor cell lines[12,36]. The results of our studies also demonstrated that it was effective both in pancreatic cancer cell line PC-2 and in breast cancer cell line MCF-7 (data not shown), suggesting that this sequence may be of universality in malignant tumors and should be a preferential sequence when study on the RNAi against surviving is carried out.

In this study, we investigated whether the sensitivity to X-irradiation could be affected by inhibiting survivin expression with siRNA. The results showed that the siRNA against survivin plasmid expression vector constructed in this study had highly specificity, and could suppress the expression of survivin in PC-2 cells efficiently suggesting that inhibiting the expression of survivin can induce spontaneous apoptosis of pancreatic cancer cells in a certain degree and enhance its radiosensitivity significantly. siRNA against survivin is of potential value in the treatment of pancreatic cancer.

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BASIC RESEARCH

Hyperlipidemia intensifies cerulein-induced acute pancreatitis associated with activation of protein kinase C in rats

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Abstract

AIM: To investigate the effects of hyperlipidemia on acute pancreatitis (AP) and the possible mechanisms.

METHODS: Rat models of hyperlipidemia and AP were established by Triton WR1339 and cerulein respectively. Human albumin was used to treat AP complicated by hyperlipidemia. In each group, we compared the histological score, volume of ascites, ratio of pancreatic wet/dry weight, serum amylase (AMY) and pancreatic acinar cell apoptosis. The level of protein kinase C (PKC) membrane translocation in pancreatic tissue was detected by Western blot.

RESULTS: In the hyperlipidemia model established by Triton WR1339, triglyceride (TG) increased remarkably and reached its peak 6 h after injection, and most rats developed mild acute pancreatitis. Histological score, volume of ascites, ratio of wet/dry weight and serum AMY in AP animals with hyperlipidemia were obviously higher than those in AP animals ($P < 0.05$) and decreased after albumin therapy but not significantly ($P > 0.05$). Apoptotic cells detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) increased in AP animals with hyperlipidemia and did not change distinctly after albumin therapy. PKC membrane translocation level increased in AP animals with hyperlipidemia and decreased remarkably after albumin therapy ($P < 0.05$).

CONCLUSION: Hyperlipidemia may induce AP or intensify pancreatic injury. Albumin therapy can not alleviate pancreatic lesion effectively. PKC activation may be one mechanism by which AP is intensified by hyperlipidemia.

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INTRODUCTION

Acute pancreatitis (AP) is a common disease that normally runs a benign course in the majority of patients. However in up to 20% of individuals the disease is severe and may be associated with a mortality of about 20%[1,2]. Since Speck in 1865[3] noted the association between hyperlipidemia and AP, their relationship has been studied by many scholars. Currently, hyperlipidemia is another main cause of AP besides cholelithiasis and alcohol abuse[4]. Serum triglyceride (TG) levels higher than 1000 mg/dl seem to be a serious factor for developing AP, particularly in patients suffering from familial hyperlipidemia (types 1, 4 or 5 according to Fredrickson's classification). AP occurs with an incidence of up to 21%[5,6]. Hydrolysis of TG by pancreatic lipase with the local release of large quantities of free fatty acids (FFAs) has been proposed as the pathogenic mechanisms. Hyperlipidemia is a common cause of AP. Moreover, lipid levels increase above normal in up to 50% of patients with AP of any cause[7].

Several studies in humans and animals have been performed to evaluate hyperlipidemia as a risk factor for developing AP. However, there is no ideal animal model of hyperlipidemic AP. Information about the influence of hyperlipidemia on the course of AP is relatively rare. The majority of investigators considered that hyperlipidemia can intensify the pancreatic lesion and systemic inflammatory response. Saharia *et al*[8] perfused the pancreas preparations with TG and FFAs (oleic acid) and found that they can lead to edema of the isolated pancreas, weight gain, and elevation of serum AMY. The injury caused by FFAs is similar to that seen with TG infusion, but it occurs more rapidly. These studies add support to the concept that hypertriglyceridemia can initiate pancreatic injury and the release of FFAs may be the mechanism. Kimuru *et al*[9] initiated an AP model with cerulein, retrograde duct injection of sodium taurocholate,

bile/pancreatic duct respectively and found that the isolate pancreatic damage is increased by TG perfusion. However, some reports suggest that hyperlipidemia could not aggravate the course of AP. Paye *et al*[10] prepared an AP model with cerulein and injected TG simultaneously, and found that histological alterations, pancreatic enzyme level and ascites in these models do not differ from those in AP patients without TG injection. To approve this result further, the authors collected very low density lipoprotein (VLDL) from AP patients and found that the VLDL intravenous infusion for hypertriglyceridemia does not alter the course of AP[11]. Therefore the role of hyperlipidemia in acute pancreatitis seems questionable.

The mechanism by which hyperlipidemia leads to AP is still unclear. A well-accepted mechanism proposed by Havel *et al*[12] is that unbound FFAs are toxic and could produce acinar cells or capillary injury. Increased concentration of chylomicrons in pancreatic capillaries causes capillary plugging and leads to ischemia and acidosis. FFAs can induce activation of trypsinogen and initiate AP. Recently, it was reported that FFAs are toxic to pancreatic β-cells leading to diabetes and insulin resistance syndrome. The toxicity is associated with abnormalities in the insulin signaling cascade and may be mediated by FFA activation of protein kinase C (PKC)[13]. Also, there is abundant PKC in pancreatic acinar cells[14]. Other similar mechanisms of FFAs may damage acinar cells.

The aim of the present work was to determine whether endogenous hyperlipidemia aggravates *in vivo* cerulein-induced edematous AP and activates PKC in pancreas of rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 250-270 g were used. Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rats were maintained at 23 ℃ in a 12 h light dark cycle, with free access to water and standard rat chow. The rats were fasted for 12 h prior to the experiment, but with free access to water. They were anesthetized with chloral hydrate (300 mg/kg intraperitoneal injection). Body temperature was monitored and maintained at 37.5 ± 1 ℃ using a heating device.

Models of hyperlipidemia, AP, and AP with hyperlipidemia

Hyperlipidemia was induced by intravenous injection of 10% Triton WR1339 solution (TyloxAPol, Sigma, USA) through the left external iliac vein. Triton WR1339, a non-ionic surface active detergent leads to an increase of endogenous TG and total cholesterol (TCH) blood concentrations[15, 16]. We determined the required amounts of Triton WR1339 with two doses of 100 mg/kg and 400 mg/kg body weight respectively. A volume of 400 mg/kg Triton WR1339 solution could induce maximal hyperlipidemia. The non-hyperlipidemic animals received an injection of physiological saline solution intravenously instead of Triton WR1339.

AP was induced by 4 h abdominal subcutaneous injection of cerulein (Sigma, American, 20 μg/kg in 0.5 mL of saline vehicle) as previously described[17].

To explore the influence of hyperlipidemia on acute experimental pancreatitis, we used five different groups of animals with 12 rats in each group. The animals of the first group [$C_{(sal)}$] received 1.0 mL saline (sal) solution, and the second group [$C_{(tri)}$] received 1.0 mL of 10% Triton WR1339 (tri) intravenously. In these two groups [$C_{(sal)}$ and $C_{(tri)}$] no pancreatitis was induced. AP was induced in the third group (AP) and the fourth group (HAP) as described above. The animals of group HAP received an injection of 1.0 mL of Triton WR1339 intravenously 6 h before pancreatitis was induced with cerulein infusion. Since albumin could effectively combine FFAs in circulation to prevent cellular damage[18], in the fifth treatment group (THAP), the rats received an injection of 1.25 g/kg albumin intravenously after pancreatitis was induced.

Analysis of serum lipid, AMY activity and histology

Blood samples were taken from all animals before and 3, 6, 9 h after induction of hyperlipidemia or AP with hyperlipidemia. The samples were used to measure TG, TCH, AMY activity.

Three and six hours after induction of AP, the rats were sacrificed by aortic puncture and blood samples taken for plasma biochemical measurements. A complete macroscopic examination of the peritoneal cavity and ascites was immediately performed, and the pancreas was removed using a standardized monobloc surgical technique. The pancreas was divided into three parts. The pancreatic head was fixed in Bouin's solution, embedded in paraffin, sliced, and stained with hematoxylin and eosin. The pancreas body was put into liquid nitrogen for PKC analysis. To better estimate the amount of edema, the remaining pancreatic tissue was weighed and dessicated for 24 h at 173 ℃ to measure its dry weight. We used the wet to dry ratio to quantify edema formation.

To quantify the histological changes, two pathologists judged the tissue samples using a point score as previously described[19,20]. Plasma AMY concentrations were determined after plasma dilution (1/20) by the colorimetric assay AMYL (Boehringer Mannhein, Germany). Plasma TG was measured using the colorimetric assay TG GPOPAP (Boehringer).

Detection of apoptosis

Recent studies indicate that mild acute pancreatitis is related to the extent of acinar cell apoptosis[21-23]. A pancreatic tissue section was prepared and assayed with the *in situ* cell death detection kit, POD (Boehringer Mannheim, Germany) following the manufacturer's instructions. Slides were counterstained with hematoxylin and viewed under a light microscope.

Activation of protein kinase C

PKC activation was determined in both cytosolic and particulate fractions as previously described[24]. Frozen pancreatic tissue(50 mg) was rapidly thawed and homogenized at 4 ℃ in lysis buffer A (mM: Tris-HCl 50; pH 7.4; NaCl 150; EDTA 2; EGTA 2; DTT 2; Na py-

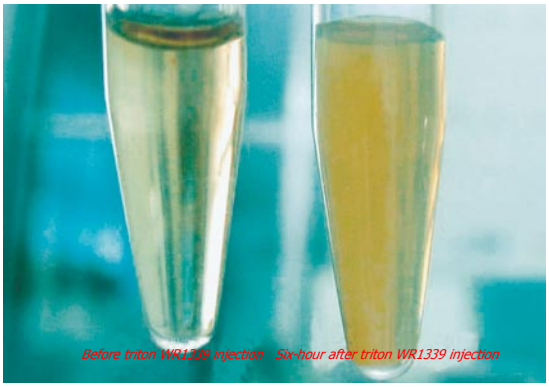


Figure 1 Development of chylomicronemia after Triton WR1339 injection.

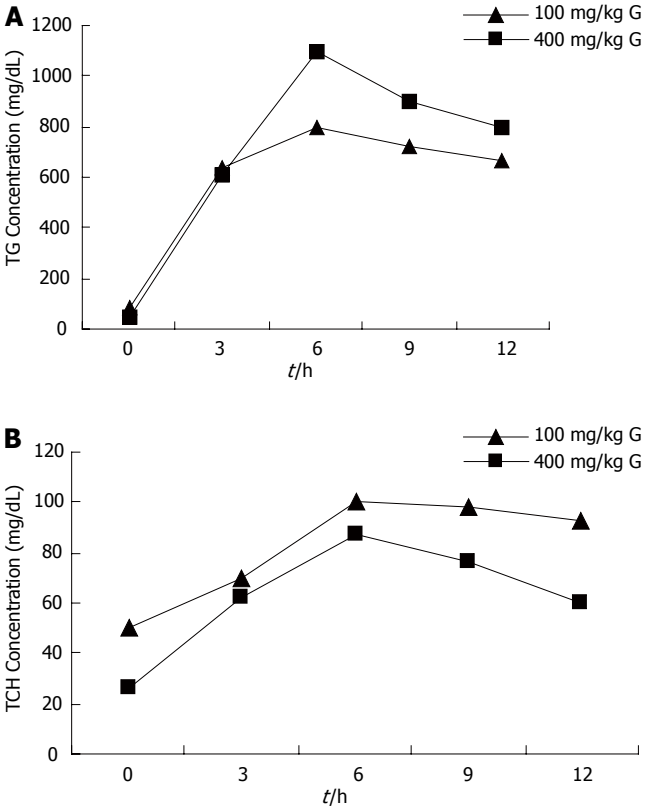


Figure 2 Curves of serum TG and TCH induced by Triton WR 1339 in rats.

rophosphate 5; KF 50; PMSF 1; 1μg/mL: Aprotinin, Leupeptin, Pepstatin A). Homogenates were centrifuged at 100 000 r/min for 30 min at 4 ℃ in an Optima TLX ultracentrifuge to yield cytosol. The pellet fractions were resuspended in 100 μL of buffer A containing 0.5% Nonidet *P*-40, sonicated and centrifuged again. The resulting supernatants taken as the particulate fractions with the cytosolic fractions were assayed for PKC immunoreactivities. Throughout the study, protein concentrations were determined by the Lowry's method with bovine serum albumin as standard. Aliquots of the cell lysates containing 100 g of total protein were resolved in 10% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences, Inc.). The membranes were blocked in TBS-Triton-X 100 (0.2% v/v, pH 7.4) containing 5% milk for 2 h at room

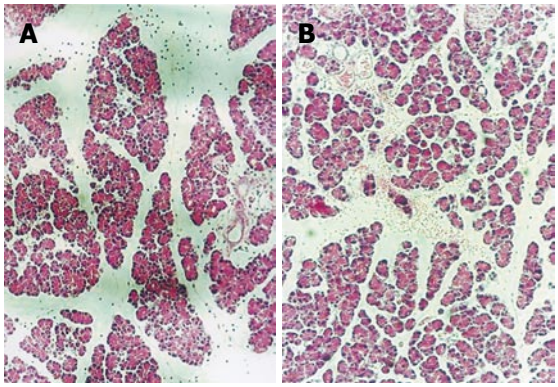


Figure 3 Microscopic pancreatic changes in rats with AP induced by cerulean (**A**) and hyperlipidemic AP induced by cerulein and Triton WR1339 (**B**) 6 h after injection (HE × 200).

temperature followed by incubation with PKC antibody at the dilution described by manufacturer's instructions at 4 ℃ overnight. The membranes were incubated with anti-goat horseradish peroxidase complex (diluted 1:2000) for 2 h at room temperature. The proteins on the membranes were exposed to X-ray films at multiple time points and visualized by ECL detection system (Amersham Biosciences, Inc.).

Statistical analysis

Data were expressed as mean ± SD and analyzed using Student's *t* test or Student's Newman Keuls-test by SPSS 11.0 software. Quantitative analysis for immunoblot was done by scanning the x-ray film and determined using the Fotodyne Gel-Pro analyzer program.

RESULTS

Changes of serum lipid, AMY and pathology of pancreas in $C_{(tri)}$ group

The animals receiving 100 mg/kg and 400 mg/kg Triton WR1339 had high TG and TCH. The serum TG increased most obviously and reached its peak 6 h after Triton WR1339 infusion with induction of chylomicronemia (Figure 1). The TG value increased 10-fold and 20-fold after 100 mg/kg and 400 mg/kg Triton WR 1339 infusion respectively, whereas the serum TCH increased only 2-fold and 3-fold (Figure 2). The serum AMY activities did not significantly increase after induction of hyperlipidemia ($P > 0.05$). The animals did not demonstrate ascites and pancreatic edema in macroscopy. Nine rats (9/12) showed mild pancreatic edema, whereas no histological changes occurred in the saline-treated animals (group $C_{(sal)}$).

Effects of hyperlipidemia on pancreatic histology in AP rats

The pancreatic changes included interstitial edema, increased interlobular interstice and infiltration of some inflammatory cells in the animals 3 h after cerulein infusion. The pancreas of AP animals 6 h after cerulein injection had serious edema with tensioned capsules and separated small lobuli. Wide lobular and acinar interstices, anamorphic acinar structure, intracytoplasmic vacuolization and

Table 1 Effects of caerulein and albumin therapy on histological pancreatic lesions, volume of ascites and ratio of pancreatic wet/dry weight (mean ± SD)

Group	Time	Histological score	Ascites(ml)	Wet/dry
C(sal)	3 h	0.33 ± 0.52	0	1.35 ± 0.09
	6 h	0.33 ± 0.52	0	1.35 ± 0.09
AP	3 h	3.50 ± 1.38[a]	0.87 ± 0.58	1.67 ± 0.05[a]
	6 h	5.33 ± 1.03[a]	1.58 ± 0.92	1.76 ± 0.14[a]
HAP	3 h	5.17 ± 1.17[c]	2.08 ± 0.58[c]	1.95 ± 0.44
	6 h	6.83 ± 1.16[c]	4.12 ± 1.58[c]	2.15 ±0.39
THAP	3 h	4.83 ± 1.16	1.87 ± 0.93	1.72 ± 0.14
	6 h	6.67 ± 1.21	3.68 ± 1.42	1.88 ± 0.33

[a]$P < 0.05$ *vs* control group, [c]$P < 0.05$ *vs* caerulein group.

Table 2 Effects of albumin therapy on serum TG, TCH and AMY in rats (mean ± SD)

Group	Time	TG	TCH	AMY
C(sal)	-	47.00 ± 23.38	26.25 ± 2.36	1658.50 ± 335.21
AP	3 h	63.66 ± 11.52	61.00 ± 6.00	5128.66 ± 259.65
	6 h	72.66 ± 22.65	67.00 ± 8.71	6641.00 ± 213.75
C(tri)	3 h	899.51 ± 240.52	76.03 ± 16.14	1652.55 ± 300.46
	6 h	797.07 ± 356.18	60.51 ± 33.49	1253.72 ± 254.80
HAP	3 h	1604.16 ± 248.10[a]	264.33 ± 50.33[a]	18390.00 ± 7158.77[a]
	6 h	1506.66 ± 327.72[c]	230.33 ± 31.87[c]	11611.66 ± 4513.67[c]
THAP	3 h	1396.83 ± 658.65	221.00 ± 60.46	20473.33 ± 12005.46
	6 h	1167.33 ± 228.28	211.00 ± 14.02	16524.39 ± 5273.52

[a]$P < 0.05$, [c]$P < 0.05$ *vs* triton group.

infiltrated inflammatory cells were observed (Figure 3). After 3 h, the pancreatic damage was intensified in the animals of group HAP, and some apoptotic bodies (inclusion) and more inflammatory cells appeared. There were scattered erythrocytes and inflammatory cells, distended small veins in the pancreas 6 h after HAP model was induced (Figure 3). There were no pathological changes after albumin therapy.

Pancreatic histological score, volume of ascites and pancreatic wet/dry ratio in rats

The animals receiving cerulein (group $C_{(tri)}$) had significantly ($P < 0.05$) higher pancreatic histological score, volume of ascites and pancreatic wet/dry ratio than the control group. The pancreatic histological score, volume of ascites and pancreatic wet/dry ratio were significantly higher ($P < 0.05$) in the AP animals with hyperlipidemia (group HAP) than in the animals receiving only cerulein infusion (group $C_{(tri)}$). After albumin treatment (group THAP) these parameters decreased slightly but were higher in the 6 h group than in the 3 h group ($P > 0.05$). These results suggested that hyperlipidemia could aggravate pancreatic edema of the AP animals and albumin therapy was not obviously effective (Table 1).

Serum TG, TCH and AMY in rats

The serum TG and TCH concentration increased 1-2 folds in the cerulein-induced AP animals. There were no statistical differences between group AP and group $C_{(sal)}$ ($P > 0.05$). The hyperlipidemic AP animals had significantly higher serum TG and TCH values than the rats receiving Triton WR1339 (group $C_{(tri)}$) injection. The serum TG and TCH concentration in the animals with albumin therapy (group THAP) decreased slightly. The AMY activities in the hyperlipidemic group $C_{(tri)}$ remained unchanged throughout the investigation, and increased obviously ($P < 0.05$) in the AP group. In comparison with the AP group, the AMY values of group HAP and THAP were significantly higher ($P < 0.05$). This result demonstrated that AP could induce mild hyperlipidemia. Albumin could not decrease the circulating lipid and AMY concentration (Table 2).

Apoptosis of pancreatic cell in rats

It was reported that the extent of acinar cell apoptosis is related to the severity of mild AP[22]. Careful biochemical and morphological examination of experimental models of AP has shown that severe AP is associated with necrosis, whereas mild acute pancreatitis is associated with apoptotic cell death[20,21]. In our study, the TUNEL methods was used for *in situ* apoptotic acinar cell identification. The apoptotic cells were not visible in the pancreas of control group, but a few apoptotic cells (brown) were visible in cerulein-induced AP animals. A subpopulation of apoptotic cells scattered throughout the tissue section was stained brown Figure 4, but the apoptotic cells did not markedly decrease after albumin therapy.

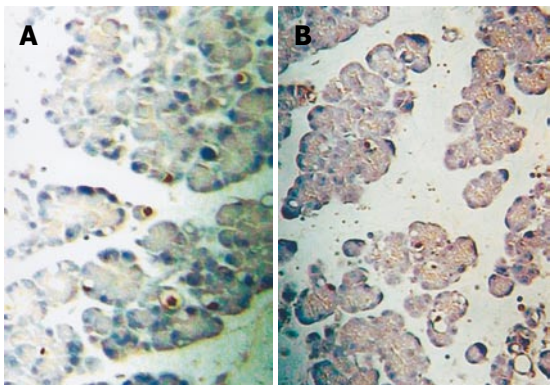


Figure 4 Detection of apoptotic cells by TUNEL in rats with AP induced by cerulein (**A**) and hyperlipidemic AP induced by cerulean and Triton WR1339 (**B**) counterstained with hematoxylin (H × 200).

Translocation of PKC in pancreatic tissue

To confirm the activation of PKC, we examined the translocation of the enzyme from the cytosol to the particulate membrane by Western blot. The cytosolic PKC gradually decreased in the control group $C_{(sal)}$, hyperlipidemic group $C_{(tri)}$, cerulein-induced AP group and HAP group. After albumin treatment the cytosolic PKC increased remarkably in group THAP. However, the particulate PKC gradually increased in the above four groups and decreased after albumin therapy (Figure 5). The degree of PKC translocation was evaluated by the proportion of particulate PKC in the total PKC as previously described[25]. The translocation rate of PKC was significantly higher ($P < 0.05$) in hyper-

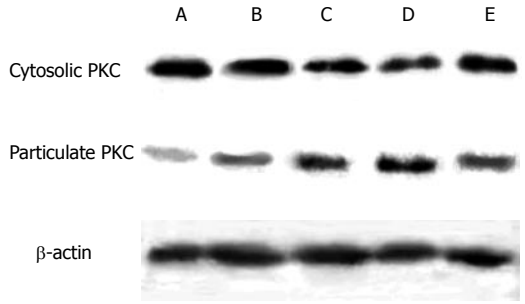


Figure 5 Effects of hyperlipidemia, AP, AP accompanying hyperlipidemia and albumin therapy on PKC membrane translocation detected by Western blot in rats of control group (A), Triton group (B), caerulein group (C), Triton+caerulein group (D), Triton+caerulein+albumin group (E).

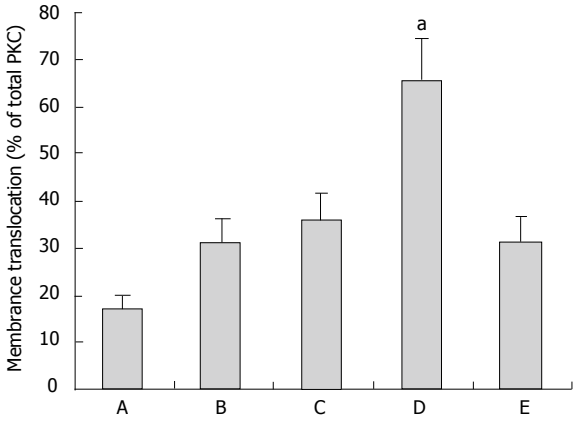


Figure 6 Effects of Triton hyperlipidemia, caerulein pancreatitis, AP with hyperlipidemia and albumin therapy on PKC membrane translocation in rats. Quantitative analysis of PKC membrane translocation level was detected by Western blot. $^{a}P < 0.05$ Triton+caerulein group *vs* caerulein and Triton+caerulein+albumin group ($n = 6$).

lipidemic AP animals (group HAP) than in non-hyperlipidemic AP rats (group AP)(Figure 6). In the hyperlipidemic AP group, the translocation rate of PKC was significantly decreased ($P < 0.01$) after albumin treatment. This result implied that hyperlipidemia could induce pancreatic PKC activation in normal and AP rats, which was consistent with the pancreatic injury. Albumin could reduce hyperlipidemia-induced PKC activation.

DISCUSSION

Hypertriglyceridemia is an etiological factor in patients with AP while TG level exceeding 1000 mg/dL or lactescent serum is a necessary condition for hyperlipidemic AP diagnosis[26]. Triton WR1339 could increase serum TG and is an ideal drug to establish hyperlipidemic animal model with TG elevation.

The rats without pancreatitis showed no clinical differences after Triton WR1339 infusion compared with saline infusion. Macroscopically, the pancreas of the hyperlipidemic and normal animals showed no difference. Microscopically, the animals treated with Triton WR1339 showed smooth edema, whereas no changes occurred in the non-hyperlipidemic animals, suggesting that severe hyperlipidemia may cause mild AP. The serum AMY activities were also slightly increased after hyperlipidemia induction, but there was no significant difference ($P > 0.05$). To investigate the effect of hyperlipidemia on AP, we established the AP model when the endogenous hyperlipidemia induced by Triton WR1339 reached its maximal value. Theoretically, significant influence of hyperlipidemia on AP would appear. We produced the hyperlipidemic AP animal model by injecting cerulein 6 h after Triton WR1339 infusion. It was reported that albumin could effectively alleviate the toxicity of TG to pancreatic cells[18]. A medium dose of albumin was injected through external iliac vein in the hyperlipidemia AP animals to observe the changes of pancreatic damage. As a result, hyperlipidemia aggravated pancreatic edema, volume of ascites and pancreatic cell apoptosis. The serum lipid in the hyperlipidemic AP animals increased compared with that in the hyperlipidemic animals, suggesting that hyperlipidemia may aggravate the course of AP while AP may exacerbate hyperlipidemia. There is a vicious circle between hyperlipidemia and AP. Accordingly, it is very important to decrease circulating lipid in clinical patients with AP. In our studies, the pancreatic damage aggravated by hyperlipidemia was not prevented by simultaneous perfusion of albumin, which might be due to the fact that the concentration of albumin in the inflamed pancreas may be too low or there may be other mechanisms of pancreatic injury. It was reported that FFAs could inhibit division and proliferation of pancreatic endocrine cells by activating PKC and induce apoptosis of pancreatic β-cells. As a result insulin secretion is decreased and diabetes occurs[27]. There is abundant PKC in pancreatic acinar cells[14]. Our study showed that FFAs could cause injury of pancreatic exocrine cells. PKC activity of pancreas increased in the hyperlipidemia and AP animals. The PKC activity changes were consistent with the degree of pancreatic injury, suggesting that PKC may participate in the course of hyperlipidemia inducing or aggravating pancreatic injury of AP. The PKC activity in hyperlipidemic AP animals was decreased significantly ($P < 0.01$) after albumin therapy, which implies that albumin may reduce the PKC activation through binding to FFAs. Although PKC activation decreased obviously ($P < 0.01$) after albumin therapy, the pancreatic injury was slightly relieved ($P > 0.05$), suggesting that PKC activation may be an important aspect of hyperlipidemia aggravating AP, and there may be other mechanisms of hyperlipidemia-induced AP.

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RAPID COMMUNICATION

Oxygen radical formation does not have an impact in the treatment of severe acute experimental pancreatitis using free cellular hemoglobin

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Abstract

AIM: Microcirculatory dysfunction and free oxygen radicals are important factors in the pathogenesis of severe acute pancreatitis. Additional oxygen delivery might enhance lipid peroxidation but may also improve pancreatic microcirculation. This study assesses the effect of free cellular bovine hemoglobin on the formation of oxygen radicals and microcirculation in a rodent model of severe acute pancreatitis.

METHODS: Fifteen minutes after induction of acute pancreatitis Wistar rats received either 0.8 mL bovine hemoglobin (HBOC-200), hydroxyethyl starch (HES) or 2.4 mL of normal saline to ensure normovolemic substitution. After 6 h of examination the pancreas was excised and rapidly processed for indirect measurement of lipid peroxidation products malondialdehyde (MDA) and reduced glutathione (GSH) in pancreatic tissue.

RESULTS: The single application of HBOC-200 improved pancreatic microcirculation and reduced histopathological tissue damage significantly. Tissue concentration of MDA did not differ between the groups. Also no differences in GSH levels were detected.

CONCLUSION: Though the single application of HBOC-200 and HES improve pancreatic microcirculation, no differences in lipid peroxidation products were detected. The beneficial effect of additional oxygen supply (HBOC-200) does not lead to enhanced lipid peroxidation.

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Key words: Hemoglobin-based-oxygen-carrier; HBOC; Blood substitutes; Severe acute pancreatitis; Free oxygen radicals; Oxidative stress

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INTRODUCTION

Microcirculatory dysfunction and free oxygen radicals are important factors in the pathogenesis of acute experimental pancreatitis[1-3]. Oxygen free radicals are formed during aerobic cellular metabolism. Under physiological conditions they are eliminated by a system of enzymatic and non-enzymatic antioxidants. Under conditions of imbalance like in acute pancreatitis between antioxidants and oxidants as in hyperoxygenation, ischemia and reperfusion and tissue inflammation "oxidative stress" occurs[4-8].

Oxygen species initiate a pronounced peroxidation of membrane lipids, detectable after 30 min by a significant rise in the lipid peroxidation marker malondialdehyde (MDA), persisting up to 16 h[8,9]. At the same time a significant decrease in glutathione thiols (GSH, potent antioxidant in the pancreas) occurs[10,11]. In the human, acute pancreatitis increases the amount of lipid peroxidation products and decreases antioxidants[12-14].

It has been shown that pancreatic microcirculatory deficits can be treated with bovine haemoglobin, which improves fluidity due to its colloid effects and delivers cell free oxygen to oxygen deprived tissue[15]. However, additional application of cell free oxygen might potentially enhance detrimental O_2 radical products[16], which could limit the positive value of bovine haemoglobin in the treatment of severe acute pancreatitis.

This study assesses the relationship between free oxygen radicals and pancreatic microcirculation in a rodent model of severe acute pancreatitis after therapeutic application of a hemoglobin based oxygen carrier (HBOC).

MATERIALS AND METHODS

The experimental protocol was approved by the Ethical

Committee of the Hamburg Federal Board of Veterinary Medicine and Animal Care. After overnight fasting with free access to water containing 20% glucose, thirty female Wistar rats (230-250 g) were randomly assigned to three groups (n = 10). Anesthesia was induced by intraperitoneal pentobarbital (40 mg/kg BW) and ketamine (10 mg/kg BW) allowing spontaneous breathing by tracheostomy. Ringer lactate was infused for fluid resuscitation keeping the central venous pressure between 4 and 6 mmHg and heart rate as well as mean arterial pressure within 10% of baseline frequency throughout the experiment (Datex AS/3 monitoring system; Hoyer, Bremen, Germany).

Acute pancreatitis was induced according to a standardised regimen[17] using a combination of intravenous cerulein infusion (5 mg/kg per hour; Takus, Pharmacia, Erlangen, Germany) at a rate of 1 mL/h over 6 h and intraductal infusion of glycodeoxycholic acid (GDOC: 10 mmol/L; 1 mL/kg for 300 s; infusion pressure 25 to 30 mmHg).

Fifteen minutes after induction the animals received either 0.8 mL bovine hemoglobin (HBOC-group) (HBOC-200, Biopure MD, USA), 0.8 mL hydroxyethyl starch (HES-group 3) or 2.4 mL of normal saline (NaCl-group) to ensure normovolemic substitution. *In vivo* fluorescence microscopy was performed with an epi-illumination unit (Zeiss, Germany). Microcirculation was observed for 6 h, measurements were performed in the head of the pancreas: 0 min was defined as the intraductal infusion of GDOC. Leucocyte adherence (area of adherent leucocytes in a percentage of the vein cross section) and functional capillary density (number of perfused capillaries to the total number of capillaries) were studied. After 6 h of examination the animals were sacrificed by an intravenous injection of pentobarbital. Pancreatic tissue samples were excised for determination of tissue damage and malondialdehyde (Lipid Peroxidation Assay Kit, Calbiochem, San Diego, USA) and GSH concentrations (Glutathion Assay Kit, Calbiochem, San Diego, USA) specified in μmol/L/g protein. The samples were immediately processed and stored at -80 ℃. The Glutathione Assay Kit takes advantage of a 2-step reaction. The first step involves the formation of thioethers when a patented reagent reacts with all mercaptans (including glutathione) in the sample. The second step is an elimination reaction that takes place in alkaline conditions. This second reaction is mediated by 30% NaOH which specifically transforms the substitution product obtained with GSH into a chromophoric thione with a maximal absorbance at 400 nm. The main advantage of the method is that it does not require any enzyme as a reagent.

The Lipid Peroxidation Assay Kit takes advantage of a chromogenic reagent (10.3 mmol/L N-methyl-2-phenylindole, in acetonitrile) which reacts with MDA and 4-hydroxyalkenals at 45 ℃. Condensation of one molecule of either MDA or 4-hydroxyalkenal with 2 molecules of 10.3 mmol/L N-methyl-2-phenylindole yields a stable chromophore with maximal absorbance at 586 nm.

Tissue protein concentration was detected by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colometric detection and quantitation of total protein. This method combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the colometric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchonic acid[18].

The histopathological scoring was analyzed by light microscopy using a validated score quantifying acinar necrosis, fat necrosis and hemorrhage, leucocyte infiltration and edema[19].

Statistical analysis

Statistical analysis of the changes from baseline values in each group of animals utilized the paired Student's t-test. Significances between the groups were assessed by the unpaired t-test. $P < 0.05$ was considered significant. All data are presented as mean ± SD.

RESULTS

Results of pancreatic microcirculation and tissue damage have been reported elsewhere[15].

Microcirculation[15]

In essence pancreatic microcirculation improved in the HBOC-200-group when compared to NaCl-group [functional capillary density (fcd): 0.7 (SD 0.3) *vs* 0.33 (0.12); $P < 0.05$]; leucocyte adherence (la): 0.27 (0.14) *vs* 0.54 (0.19); $P < 0.05$). HES also improved pancreatic microcirculation when compared to NaCl-group (fcd: 0.49 (0.15) *vs* 0.33 (0.12); $P < 0.05$ and la: 0.39 (0.11) *vs* 0.54 (0.19); $P < 0.05$).

Tissue damage[15] (Figures 1-4)

The histological score showed significantly less tissue damage in HBOC-group (Figure 1) versus NaCl-group (6.25 *vs* 9.25) (range 3-8.5 *vs* 8-10.75); $P < 0.001$. The overall score is reflected within all subgroups. Comparing HES and HBOC-therapy significantly less tissue damage was found (6.25 *vs* 8) (range 3-8.5 *vs* 6.5-10.25); $P = 0.006$ in the HBOC group. When comparing HES (Figure 2) and NaCl (Figure 3) less necrosis and hemorrhaging (1.75 *vs* 2.75) (range 1.5-2.75 *vs* 2.25-3.5) was found in the HES therapy group(Figure 4).

Oxygen radicals

Tissue concentration of MDA did not differ between the groups (HBOC-200-group: 4.37 ± 1.11 μmol/L per g protein; HES-group: 3.77 ± 1.45 μmol/L per g protein; NaCl-group: 4.31 ± 1.19 μmol/L per g protein. MDA-tissue levels (n = 10) of sham operated rats were 5.41 ± 2.12 μmol/L per g protein.

Also no differences in GSH levels were detected HBOC-group: 2.93 ± 1.72 μmol/L per g; HES-group: 4.46 ± 3.55 μmol/L per g; NaCl-group: 3.7 ± 3.5 μmol/L per g. Control GSH-tissue levels (n = 10) of sham operated rats were 12.19 ± 7.04 μmol/L per g protein (Figure 5).

DISCUSSION

Clinical investigations with free hemoglobin solutions have

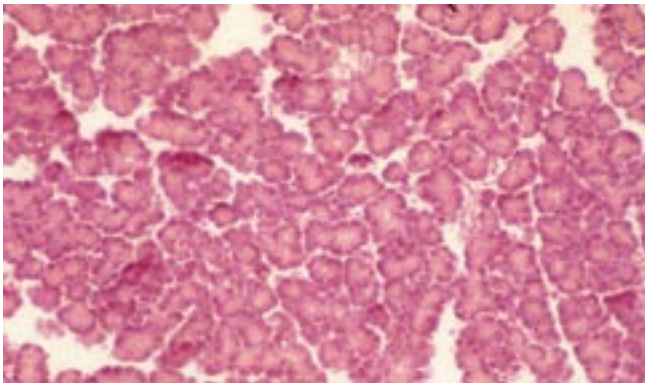


Figure 1 Light microscopy (display alternation = 200) of pacreatic tissue 6 h after induction of acute pancreatitis and traement with HBOC (score: 6 points out of 16).

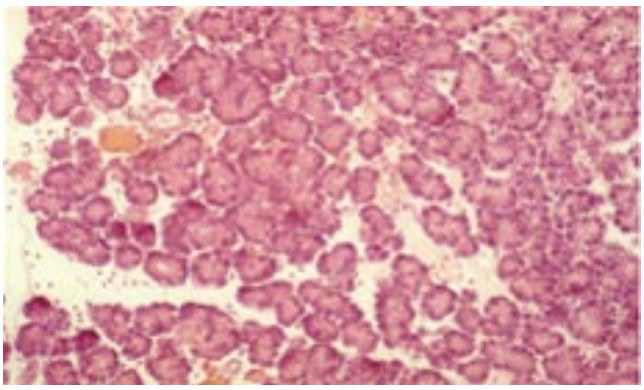


Figure 2 Light microscopy (display alternation = 200) of pacreatic tissue 6 h after induction of acute pancreatitis and traement with HES (score: 8.5 points out of 16).

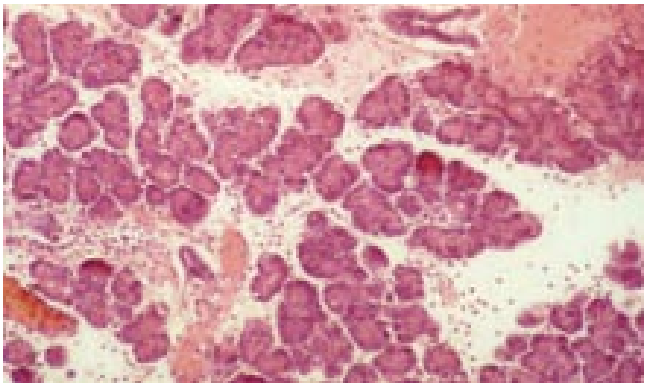


Figure 3 Light microscopy (display alternation = 200) of pacreatic tissue 6 h after induction of acute pancreatitis and treatment with NaCl (score: 10 points out of 16).

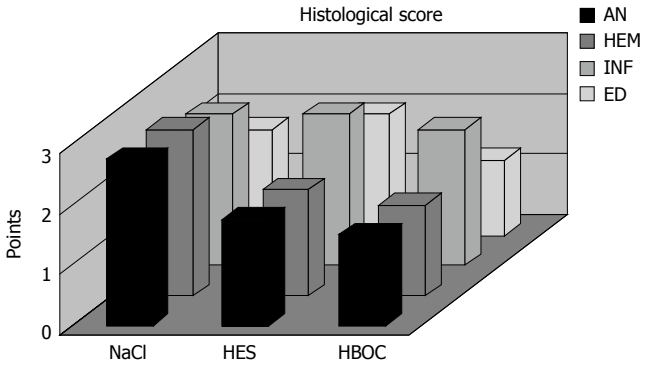


Figure 4 Histological scoring of pancreatic tissue 6 h after induction of acute pancreatitis. Values are mean ± range. (AN = acinar necrosis, HEM = hemorrhage, INF = leucocyte infiltration, ED = edema).

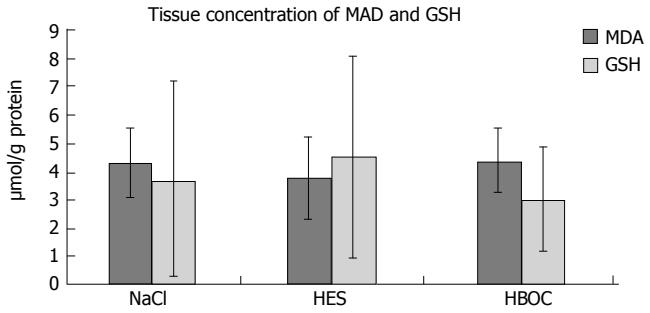


Figure 5 Tissue concentration of MDA and GSH in pancreatic tissue 6 h after induction of acute pancreatitis. Values are mean ± SD.

been performed since the late 1800 s. Over the years complications like renal toxic effects and short circulation time have been reduced. The new blood substitutes are able to replace blood, colloid and crystalloid solutions act as oxygen transporter in many experimental settings. The ability of potent oxygen transport to oxygen deprived tissue has been shown in different animal models[15,20].

Vincent *et al* investigated the influence of heme-binding proteins in heme-catalyzed oxidations. They confirmed previous studies that heme acts as a catalyst of H_2O_2-dependent lipid peroxidation[21]. It has been shown that diene conjugation caused by oxygen radicals gives an adequate assessment of lipid peroxidation in tissue extracts. Several studies emphasize the role of oxygen free radicals in the pathogenesis of experimental acute pancreatitis[1,3,22-26]. Oxygen radicals are generated by different biological systems. Sources are reperfusion injury as well as the respiratory burst of PMN-leucocytes[27-30]. Local tissue damage has successfully been treated by the use of radical scavengers[31,32]. However increased heme and oxygen availability like in HBOC-therapy might lead to higher generation of oxygen radicals and tissue damage caused by lipid peroxidation.

A single application of a hemoglobin based oxygen carrier in this model of severe acute pancreatitis reduces the tissue damage and ensures a stable microcirculation. Colloids like HES and Dextrane have also been proven to be an effective therapy for microcirculatory dysfunctions[33-36]. The superiority of HBOC[34,36,37] might be explained by the combining effect of a colloid and noncorpuscular oxygen carrier. HBOC releases oxygen in areas of poor perfundation and ensure tissue oxygenation.

However, the hypothesis that hyperoxygenation and additional oxygen supply might lead to an increased production of oxygen free radicals was not supported by this data. This study could not confirm former publications suggesting that increased heme availability promotes the enhencement of lipid peroxidation. Dunne *et al* demonstrated the reactivity of various modified hemoglobins to hydrogen peroxide in terms of free radical formation. They compared PHP hemoglobin (crosslinked between the β-subunits

and conjugated with polyoxyethylene) with DBBF hemoglobin (crosslinked between the β-subunits using dibromosalicylfumerate, and control HbA_0. All the blood substitutes generated free radicals. MDA- levels were equal in nearly all groups. When compared to sham operated animals MDA-levels in the HBOC groups reached the same levels. One explanation of this finding could be that the level of MDA and GSH might have reached their peak within the first hours of pancreatitis and decreased to normal levels after 6 h. This theory is underlined by former studies[23,32,38-39] Schoenberg *et al* determined the peroxidation products, conjugated dienes and MDA in hemorrhagic pancreatitis induced by retrograde injection of sodium-taurocholate . The tissue levels of MDA increased, reaching their highest level after 1 h. Two hours later the lipid peroxides had returned to control levels.

GSH-tissue levels also showed no statistically relevant differences in the groups. Compared to GSH-levels of sham operated rats a decrease was found which might be an indication for oxidative stress in all groups. Dabrowski *et al* were able to show a decrease in pancreatic GSH at an early stage of cerulein-induced pancreatitis in rats[40,41].

Other researchers were able to prevent local tissue damage in experimental pancreatitis by the use of radical scavenging therapy[26,31-32]. Since we could not detect differences in oxidative stress markers we would explain the reduced histopathological damage by an improved pancreatic microcirculation. An excess formation of free oxygen radicals in the setting of additional oxygen supply was not detected.

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RAPID COMMUNICATION

MRCP and MRI findings in 9 patients with autoimmune pancreatitis

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Abstract

AIM: To evaluate magnetic resonance cholangiopancreatography (MRCP) findings in conjunction with magnetic resonance (MR) images in autoimmune pancreatitis (AIP) patients.

METHODS: Nine patients with AIP underwent MRI, MRCP, endoscopic retrograde cholangiopancreatography (ERCP), computed tomography, and ultrasonography. The MRCP and MR images taken before and after steroid therapy were reviewed and compared with other imaging modalities. The MRCP findings of the AIP cases were compared to those of 10 cases with carcinoma of the head of the pancreas.

RESULTS: On MRCP, the narrowed portion of the main pancreatic duct noted on ERCP was not visualized, while the non-involved segments of the main pancreatic duct were visualized. The degree of upstream dilatation of the proximal main pancreatic duct was milder than that seen in cases of pancreatic carcinoma. Stenosis or obstruction of the lower bile duct was detected in 8 patients. MR images showed enlargement of the pancreas with decreased signal intensity on T1-weighted MR images, increased signal intensity on T2-weighted MR images, and, in 3 patients, a hypointense capsule-like rim. After steroid therapy, the previously not visualized portion of the main pancreatic duct was seen, along with improvement of the bile duct stenosis. Pancreatic enlargement decreased, and the abnormal signal intensity on both T1- and T2-weighted MR images became isointense.

CONCLUSION: MRCP cannot differentiate irregular narrowing of the main pancreatic duct seen with AIP from stenosis of the main pancreatic duct seen with pancreatic carcinoma. However, MRCP findings in conjunction with MR imaging of pancreatic enlargement that shows abnormal signal intensity on T1- and T2-weighted MR images are useful in supporting a diagnosis of AIP.

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Key words: Autoimmune pancreatitis; Magnetic resonance cholangiopancreatography; Magnetic resonance

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INTRODUCTION

Autoimmune pancreatitis (AIP) has recently been described as a peculiar type of chronic pancreatitis with underlying autoimmunity[1-5]. Patients with AIP almost always have enlargement of the pancreas[1-5], and their major presenting symptom (65%[6]-68%[7]) is obstructive jaundice due to obstruction of the bile duct. Since, pancreatic or bile duct carcinoma is suspected, pancreaticobiliary duct system is examined by endoscopic retrograde cholangiopancreatography (ERCP). The hallmark finding of AIP on direct pancreatogram is irregular narrowing of the main pancreatic duct; stenosis of the intra- or extrahepatic bile duct is frequently detected on cholangiography[1,2,5-7].

Magnetic resonance cholangiopancreatography (MRCP) has become popular as a non-invasive method for obtaining high quality images of the pancreaticobiliary tree[8]. Although the features of AIP on computed tomography (CT) and ERCP have been reported, the MRCP features of AIP have not been clarified. The aim of this study was to describe the MRCP findings in AIP patients before and after steroid therapy and to review the utility of MRCP for the diagnosis for AIP.

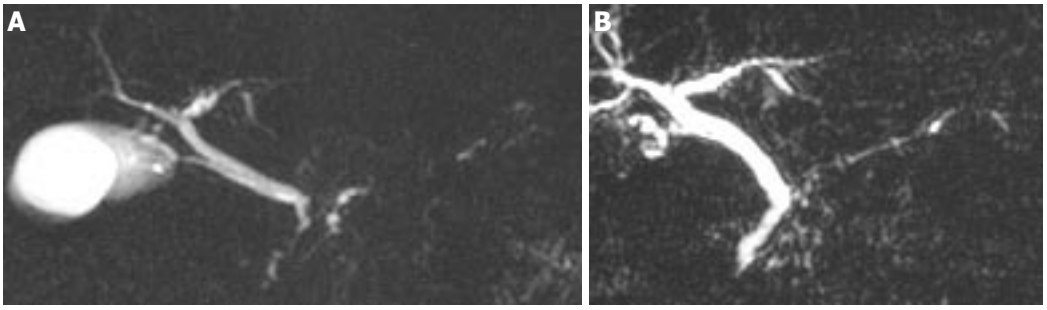


Figure 1 A: On MRCP of a patient with autoimmune pancreatitis, 2 segmentally skipped lesions of the main pancreatic duct in the head and body of the pancreas were not visualized; **B:** After steroid therapy, the entire MPD was seen on MRCP.

Table 1 ERCP and MRCP findings of patients with autoimmune pancreatitis

ERCP	MRCP
Diffuse narrowing of MPD in entire pancreas (n = 1)	Non-visualized MPD in entire pancreas (n = 1)
Segmental narrowing of MPD in the head of pancreas (n = 3)	Non-visualized MPD in the head of pancreas (n = 3)
Two skipped narrowing of MPD in the head and body of pancreas (n = 3)	Two skipped non-visualized MPD lesions in the head and body of pancreas (n = 3)
Segmental narrowing of MPD in the tail of pancreas (n = 2)	Non-visualized MPD in the tail of pancreas (n = 2)

MPD: main pancreatic duct.

MATERIALS AND METHODS

Subjects

Nine patients (6 males and 3 females, average age 60.3 years) with AIP who had MRCP in the time period from 1997 to 2005 were enrolled in this study. All patients had ERCP, ultrasonography (US), and CT. ERCP showed irregular narrowing of the main pancreatic duct in all patients [diffuse (n = 1), segmental-head (n = 3), segmentally skipped-head and body (n = 3), segmental-tail (n = 2)]. CT and US showed enlargement of the pancreas in 8 patients [diffuse (n = 3), segmental-head (n = 3), segmental-tail (n = 2)]. Six patients had elevations of serum IgG, 7 patients had elevations of serum IgG4, and 3 patients had antinuclear autoantibody. Six patients presented with obstructive jaundice. On cholangiography, stenosis of the bile duct was detected in 8 patients [lower common bile duct (n = 5) and intrahepatic and lower common bile duct (n = 3)]. All patients were given steroid treatment and responded both morphologically and serologically. From the radiological and serological findings, in addition to effectiveness of steroid treatment, all 9 patients were diagnosed as having AIP.

Methods

MRCP was done using a 1.5 T magnetic resonance imaging (MRI) machine (INTERA, PHILIPS Co Ltd, Netherlands) by coronal heavily T2-weighted single-shot rapid acquisition with relaxation enhancement. The MRCP and MR images taken before and after steroid therapy were reviewed and compared with other imaging modalities. The MRCP findings of the AIP cases were compared to those of 10 cases with carcinoma of the head of the pancreas.

RESULTS

The narrowed portion of the main pancreatic duct seen on ERCP was not visualized on MRCP, while the non-involved segments of the main pancreatic duct were seen on MRCP. On MRCP, the entire main pancreatic duct was not seen in 1 patient in whom the main pancreatic duct showed diffuse narrowing on ERCP. In 3 patients in whom the main pancreatic duct was segmentally narrowed in the head region on ERCP, the MRCP visualized the main pancreatic duct only in the body and tail of the pancreas, but not in the head region. In 2 patients, MRCP showed the main pancreatic duct only in the head and body of the pancreas, but not in the tail region. On MRCP, 2 segmentally skipped lesions of the main pancreatic duct in the head and body of the pancreas were not visualized in 3 patients (Figure 1A and Table 1). Although upstream dilatation of the proximal main pancreatic duct was detected in 6 patients, the degree of dilatation was milder than that in cases of pancreatic carcinoma. Stenosis or obstruction of the lower bile duct was detected in 8 patients, of whom 3 patients also showed stenosis of the intrahepatic bile duct accompanied by dilatation of the proximal bile duct, similar to the findings on ERCP.

All patients showed enlargement of the pancreas with abnormal signal intensity on T1- and T2-weighted images, though the degree of abnormal intensity was different among the cases. On T1-weighted MR images, the signal intensity of the affected pancreatic lesion was decreased compared to the signal intensity in the liver (Figure 2A). T2-weighted MR images showed increased signal intensity in the pancreatic parenchyma compared to that of the liver (Figure 2B). A capsule-like rim, which was hypointense on both T1- and T2-weighted images, was detected in 3 patients.

After steroid therapy, the portion of the main pancreatic duct that was previously not visualized on MRCP was seen (Figure 1B), and an improvement of the upstream dilatation of the proximal main pancreatic duct was evident. Stenosis or obstruction of the lower or hilar bile duct was found to be improved. The enlarged pancreas became normal in size (n = 6) or atrophic (n =



Figure 2 **A:** On T1-weighted MR images of a patient with autoimmune pancreatitis, the signal intensity of the affected pancreatic lesion was slightly decreased compared to the signal intensity in the liver; **B:** T2-weighted MR images showed increased signal intensity in the pancreatic parenchyma than that of the liver with a capsule-like rim which was hypointense; **C:** After steroid therapy, the enlarged pancreas became atrophic on T1-weighted MR images.

3) after steroid therapy. In all patients on both T1- and T2-weighted MR images, the abnormal signal intensity improved to being isointense with that of the liver, and the capsule-like rim disappeared (Figure 2C).

DISCUSSION

Since Yoshida *et al*[9] first proposed the existence of AIP in 1995, many cases of AIP have been reported. This unique form of chronic pancreatitis appears to be a distinct disease entity. AIP occurs predominantly in elderly males and frequently presents as obstructive jaundice[1,2,5-7]. This presentation is also typical of pancreatic carcinoma. As most patients with AIP respond to oral steroid therapy, an accurate diagnosis of AIP is necessary to avoid unnecessary laparotomy or pancreatic resection. Recently, MRCP has become a viable alternative to diagnostic ERCP as a non-invasive method for obtaining high quality images of the pancreaticobiliary tree[8]. We compared MRCP findings and MR images before and after steroid therapy in AIP patients.

The characteristic radiological images of AIP include irregular narrowing of the main pancreatic duct on ERCP and enlargement of the pancreas on CT and US[1-3,5-7]. The irregular narrowing of the main pancreatic duct on direct pancreatography, which is usually longer than the stenosis of the main pancreatic duct detected in patients with pancreatic carcinoma, is one of the useful findings that differentiate AIP from pancreatic carcinoma[1,3,10]. In the present study, the narrowing of the main pancreatic duct on ERCP was not visualized on MRCP, while the other region of the main pancreatic duct was visualized. After steroid therapy, the previously unvisualized lesions of the main pancreatic duct were seen. Thus, non-visualization of the main pancreatic duct might suggest narrowing of the main pancreatic duct. MRCP cannot differentiate irregular narrowing of the main pancreatic duct from stenosis of the main pancreatic duct in pancreatic carcinoma; thus, ERCP is necessary to diagnose AIP. However, MRCP findings of skipped, non-visualized main pancreatic duct lesions, accompanied by a lesser degree of upstream main pancreatic duct dilatation than that seen in pancreatic carcinoma, might suggest the presence of AIP. The MRCP of AIP patients frequently showed stenosis of the intra- or extrahepatic bile duct. T1-weighted MR images of AIP patients showed enlargement of the pancreas with decreased signal intensity, while T2-weighted MR images showed increased signal intensity, sometimes associated with a hypointense capsule-like rim in the involved segments. In previous reports, a decreased signal intensity on T1-weighted MR images, reflecting fibrosis in the pancreatic parenchyma, has been reported. Irie *et al*.[11] noted the hypointense capsule-like rim in the involved segment on T2-weighted MR images; this appears to be a characteristic finding of AIP, and is thought to be related to an inflammatory process involving peripancreatic tissue.

There have been no previous reports dealing with the change in the intensity in the pancreas of patients with AIP after steroid therapy. In the present study, on both T1- and T2-weighted MR images, abnormal signal intensity of the pancreas improved to isointensity with that of the liver. The enlargement of the pancreas and the capsule-like rim disappeared. Thus, MRCP and MR images are useful for evaluating the effectiveness of steroid therapy.

In conclusion, MRCP cannot differentiate the irregular narrowing of the main pancreatic duct seen with AIP from the stenosis of the main pancreatic duct seen with pancreatic carcinoma. However, the MRCP findings of skipped, non-visualized main pancreatic duct lesions, accompanied by a lesser degree of upstream dilatation of the main pancreatic duct, might suggest the presence of AIP. These MRCP findings, in conjunction with MR images of pancreatic enlargement showing abnormal signal intensity on T1- and T2-weighted MR images, are useful in supporting a diagnosis of AIP.

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RAPID COMMUNICATION

Incidence and predictors of upper gastrointestinal bleeding in patients receiving low-dose aspirin for secondary prevention of cardiovascular events in patients with coronary artery disease

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Abstract

AIM: The use of low-dose aspirin to prevent cardiovascular disease events is well established. However, the incidence and predictors of upper gastrointestinal bleeding (UGIB) with its use are unknown. We studied prospectively the incidence and outcome of peptic ulceration in low-dose aspirin users.

METHODS: A total of 991 patients with coronary artery disease (CAD) on low-dose aspirin were prospectively followed-up for two years for the occurrence and clinical features of first hospitalized episode of UGIB.

RESULTS: UGIB had a bimodal presentation with 45% occurring within four months of aspirin initiation and had an overall prevalence of 1.5% per year. There was no UGIB-related death. Hypertension (*OR* = 4.6, 95%CI 1.5 - 14.7, *P* = 0.009), history of peptic ulceration (*OR* = 3.1, 95%CI 1.1 - 9.0, *P* = 0.039), tertiary education (*OR* = 3.08, 95%CI 1.1 - 9.0, *P* = 0.039) and higher lean body mass (*P* = 0.016) were independent factors associated with UGIB. Use of nitrate did not reduce UGIB.

CONCLUSION: The incidence of UGIB in patients with CAD on long-term low-dose aspirin is low, but is accompanied with significant morbidity. With prolonged use of aspirin, UGIB continues to be a problem for those with risk factors and especially in patients with a history of peptic ulcers, in which UGIB tends to occur early after aspirin therapy.

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Key words: Coronary artery disease; Aspirin; Gastrointestinal bleeding

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INTRODUCTION

Anti-platelet therapy with low-dose aspirin (75-325 mg) reduces the risk of vascular events in patients with established coronary artery disease (CAD)[1-3]. However, the use of aspirin, even at a low dose for secondary prevention of cardiovascular events, is associated with a small but significant increase in the risk of upper gastrointestinal bleeding (UGIB). In addition, previous studies have also reported several risk factors, such as the use of non-steroidal anti-inflammatory drugs (NSAIDs) and old age, implicated in the development of UGIB in patients taking aspirin[4-8]. However, the incidence of aspirin-related UGIB in patients with CAD for the secondary prevention of cardiovascular events and the time frame of UGIB occurrence are uncertain. In addition, effects of several emerging therapies, such as the use of dual antiplatelet agents, nitrate or ulcer-protecting drugs for the development of UGIB, have not been systematically evaluated.

In a cohort of patients with established CAD recruited into a Cardiac Rehabilitation Programme, we prospectively monitored the time of occurrence of UGIB in patients taking low-dose aspirin for the secondary prevention of

cardiovascular events over two years. We also sought to determine the possible predictive factors associated with UGIB.

MATERIALS AND METHODS

Study population

In this prospective study, we recruited all patients with established ischemic heart disease (myocardial infarction, angina pectoris, angiographic-proven coronary artery disease) referred to a Cardiac Rehabilitation Programme in Tung Wah Hospital (an University-affiliated hospital) between September 1997 and April 2002. All the patients were started on low-dose aspirin (75-300 mg) according to referring physicians' discretion. Apart from collecting data on demographics, other parameters related to cardiac rehabilitation like education level, functional class, left ventricular function, exercise capacity, body mass index, body fat content, body lean mass content, medications used were also recorded. A database was specially designed to collect the required information prospectively. Patients were followed-up once every 3-4 mo and the occurrence of UGIB was noted. Detailed information about each UGIB event was obtained from interpretation and review of hospital records.

Upper gastrointestinal bleeding

The occurrence of UGIB was defined as the clinical presentation of hematemesis or passage of melena or unexplained drop in hemoglobin of $\geqslant$ 20 g/L. Upper gastrointestinal endoscopy with therapeutic intervention(s) to obtain haemostasis (using epinephrine injection or heater probe application, when needed) was performed in all these patients presented with UGIB. *Helicobacter pylori* (*H pylori*) status was determined using a 24-h rapid urease test on antral biopsy specimens and histological detection of *Helicobacter*-like organisms using the haematoxylin and eosin stain, and Giemsa stain on biopsies taken from the gastric antrum and/or body. The rapid urease test at 24 h has been validated in our hospital with a sensitivity and specificity of 99% and 100%, respectively[9]. In addition, the use of histological examination of these biopsy specimens, the *H pylori* status was correctly evaluated even in patients with active UGIB, with the understanding that rapid urease test would be rendered inaccurate at those situations.

Statistical analysis

Univariate analyses were performed, using Student's *t* test and Mann-Whitney *U* test for continuous variables and Chi-square test or Fisher's exact test for categorical variables to assess the risk factors associated with UGIB. A multiple logistic regression model was designed to determine the factors (age, gender, educational level, occupation, hypertension, diabetes mellitus, renal impairment, history of peptic ulcer diseases, lean body mass, body fat, obesity, New York Heart Association (NYHA) functional class, left ventricular function, use of dual anti-platelet agents/NSAID/ulcer protective drug) associated with UGIB. To find the best model, a backward elimination stepwise procedure was carried out such that the factor would be eliminated from the analysis if the corresponding *P* value was greater than 0.15. A two-tailed *P* value of 0.05 or less was considered statistically significant.

Table 1 Baseline characteristics of 991 patients with coronary artery disease started on low-dose aspirin (mean ± SD, *n*, %)

	No UGIB (*n* = 962)	UGIB (*n* = 29)	*P* values
Age (mean ± SD)	64 ± 11	67 ± 11	0.663
Sex (M/F)	693/268	23/6	0.683
Hypertension (%)	519 (54)	22 (76)	0.008
Diabetes mellitus (%)	342 (35.5)	10 (34.5)	0.906
Renal impairment (Cr > 200 umol/L) (%)	42 (4.4)	5 (17.2)	0.001
Serum creatinine	120 ± 93	158 ± 103	0.002
History of MI (%)	536 (56)	16 (55)	0.095
History of PU (%)	74 (7.7)	9 (31.0)	0.006
LVEF < 40% (%)	388 (40.3)	11 (37.9)	0.034
NYHA (III-IV) (%)	82 (8.5)	6 (20.7)	0.048
% Body fat	34 ± 7	30 ± 7	0.036
% Lean body mass	66 ± 8	70 ± 7	0.049
Education level (tertiary) (%)	107 (11.1)	5 (17.2)	0.05
Nitrate use (%)	540 (56.1)	21 (72.4)	0.09

Cr: creatinine; MI: myocardial infarction; PU: peptic ulcer; LVEF: left ventricular ejection fraction; NYHA: New York Heart Association functional class.

RESULTS

Characteristics of patients and incidence of upper gastrointestinal bleeding (Table 1)

Between September 1997 and April 2002, a total of 991 patients with established ischemic heart disease were enrolled into the study and followed-up prospectively for two years. Of these, 716 (72.3%) were male (mean age, 64.8 ± 11 years); 552 (55.7%) had history of myocardial infarction (MI); 541 (54.8%) were hypertensive; 352 (35.5%) had diabetic mellitus; and 546 (54.7%) patients were smokers. Nitrate use was found in 561 (57.1%) patients. One hundred and twelve patients (11.3%) had received tertiary education. Twenty-nine patients developed UGIB over a follow-up period of two years, giving the incidence of 1.5% per year. For those who had a bleeding event, 72% did not have new-onset ulcer pain; 52% required blood transfusion (mean, 3.3 units; range, 1-8 units); 62% with a drop in hemoglobin of greater than 3 g/dL; 24% of these patients required more than one therapeutic upper gastrointestinal endoscopies for haemostasis. Peptic ulceration was the main cause of UGIB in our series (gastric ulcer, 38%; duodenum ulcer, 52%); aspirin-related hemorrhagic gastritis did not occur in our cohort. *H pylori* positivity status was present in 50% of patients presented with UGIB.

Of those who had UGIB, 21% of all the events occurred in the first month and 45% occurred in the first 4 mo after initiation of low-dose aspirin (early bleeders) (Figure 1). The 'late bleeders' (55%) developed UGIB at a mean of 20.8 ± 5.3 mo after initiation of therapy, with 88% (14/16) of them bled at least once after one year of aspirin therapy. In addition, about a third of those

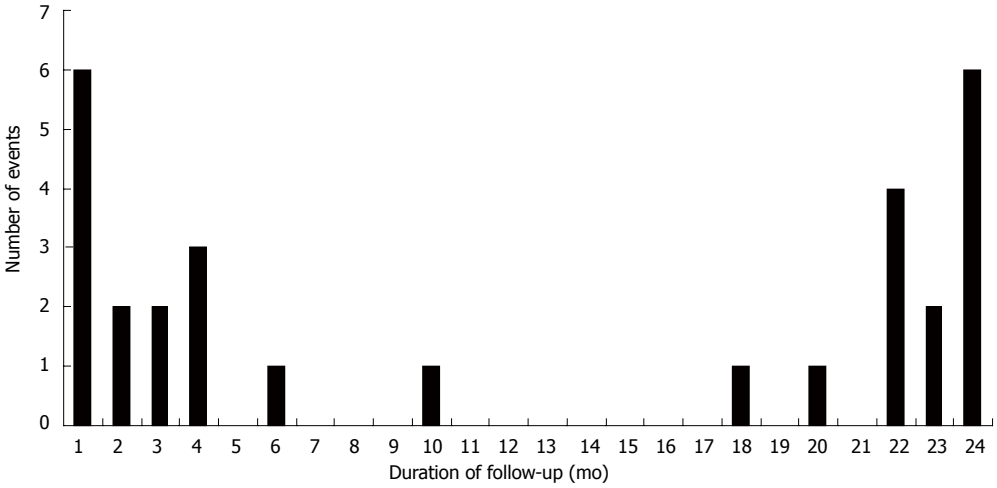


Figure 1 Bimodal presentation of upper gastrointestinal bleeding events in a two-year follow-up period.

who presented with UGIB (9/29) had a history of peptic ulcer/GIB/gastrectomy, in which 56% (5/9) of them were early bleeders. This indicates a 1:8 chance of having UGIB in patients with history of peptic ulceration while started on low-dose aspirin, which have a tendency to have early bleeding event; 97% of patients with UGIB required hospitalization. Death or myocardial infarction did not occur during the UGIB episodes and no patient required surgery for treatment of the UGIB. Nitrate use was recorded in 62% of the bleeders; 21% of those who had an UGIB were on ulcer-protecting drugs during presentation, but only 10% of them reported concurrent use of NSAID.

Predictors of UGIB

Univariate analyses showed that the presence of hypertension (*OR* = 3.1, 95%*CI* 1.3 - 7.7, *P* = 0.008), renal impairment (*OR* = 4.8, 95%*CI* 1.7 - 13.1, *P* = 0.001), history of peptic ulcer (*OR* = 4.6, 95%*CI* 2.0 - 10.7, *P* = 0.002), patients with impaired left ventricle function (EF < 40%) (*P* = 0.034) or NYHA functional class III-IV (*OR* = 3.0, 95%*CI* 1.2 - 7.6, *P* = 0.048), patients with high lean body mass (70% ± 7% *vs* 66% ± 8%, *P* = 0.049) or low body fat content (30% ± 7% *vs* 34% ± 7%, *P* = 0.036) and patients with tertiary education (*OR* = 3.1, 95%*CI* 1.1 - 9.0, *P* = 0.05) were predictors of the development of UGIB. Use of anti-ulcer therapy (in small number of patients) was not associated in prevention of UGIB. The use of nitrate did not reduce the risk of UGIB (*P* = 0.09). However, multivariate analyses using a logistic regression model demonstrated that only patients with hypertension (*OR* = 4.63, 95%*CI* 1.5 - 14.7, *P* = 0.009), history of peptic ulcer (*OR* = 3.1, 95%*CI* 1.1 - 9.0, *P* = 0.039), tertiary education (*OR* = 3.1, 95%CI 1.1 - 9.0, *P* = 0.039) or with high lean body mass (707% ± 7% *vs* 667% ± 8%, *P* = 0.0157) had a higher probability of UGIB while taking low-dose aspirin for secondary prevention of cardiovascular events (Table 2).

Table 2 Multivariate analysis of predictors of upper gastrointestinal bleeding in patients with coronary artery disease on low-dose aspirin (mean ± SD)

	95%*CI*	Odds ratio	*P* values
Education level (tertiary)	1.05 - 8.98	3.08	0.039
Hypertension	1.46 - 14.74	4.63	0.009
History of peptic ulcer	1.05 - 9.00	3.08	0.039
% Lean body mass (body fat content)			0.0157

DISCUSSION

To the best of our knowledge, this is the first prospective study for the first UGIB in patients started with low-dose aspirin for secondary prevention of cardiovascular events. UGIB occurred at a rate of 1.5% per year, and was due to peptic ulceration that required hospitalization and blood transfusion. Twenty-four percent of those with UGIB required more than one therapeutic endoscopy to secure haemostasis. On the other hand, new-onset ulcer syndrome was not present in majority of those patients that developed an UGIB. These data also showed that when patients started with low-dose aspirin and bled subsequently, 21% of them had the event in the first month of treatment, 45% of all bleeding events occurred in the first 4 mo of starting low-dose aspirin; whereas most of the other bleeding events took place after at least one year of therapy. This observation implies that not only the risk of UGIB associated with low-dose aspirin use does not decrease with time, rather, there exists a bimodal presentation of the event in our series with one peak occurring in the early phase of starting treatment and the second occurred beyond the first year. This is in contrary to previous studies, in which the rate of UGIB occurred in a constant fashion throughout the entire period of study[4,8]. There are several possible explanations for the difference. Unlike other studies, we prospectively recorded first episode of UGIB after aspirin initiation instead of recruiting those presenting with the event[5]. In addition, others reported the use of a higher-dose of aspirin for the prevention of cardiovascular events[6]. Furthermore, some animal studies reported the possibility of adaptation of the gastric mucosa to aspirin therapy with time, and this might explain why patients begin to have less bleeding after receiving several mo of aspirin therapy[14].

We demonstrated the predictors of UGIB events for patients taking low-dose aspirin. Better-educated people

were more likely to experience UGIB (*OR* = 3.1, the odds of UGIB was about 3 times more among people who received tertiary education than those who received secondary education or less). One explanation is that better educated people might be more compliant to prescribed medications, and they generally have much higher pressure/stress at work, and hence have a higher incidence of UGIB[10-11]. Consistent to previous reports, patients with a past history of peptic ulcer problems or UGIB were still at high risk of developing UGIB despite the use of low-dose aspirin[4]. Furthermore, we showed that most of them bled early on initiation of aspirin and thus deserve treatment for ulcer prophylaxis. In our study, patients with hypertension had the highest odds ratio for developing UGIB, concur with the traditional association between hypertension and the development of peptic ulcer[15]. Another interesting finding was that patients with a higher lean body mass had a higher probability for having an UGIB. A recent report suggested that obese patients are more prone to the development of aspirin resistance[12], so whether non-obese patients with slender-build (and hence a higher lean body mass content) are more susceptible to aspirin effect, including its toxicity on the stomach, will need further evaluation. Unlike a previous report[4], the use of nitrate was not linked to a reduction of UGIB in our study (P = 0.09). This may be due to a purely statistical problem as a large majority (57.1%) of the CAD patients in our cohort were on nitrate, which might mask the protective effect, if any of the latter; alternatively, other confounding factors, such as hypertension, leaner subjects in our series, might negate the effect of nitrate. The presence of renal impairment or heart failure/left ventricular dysfunction was believed to be predictors of UGIB as reported in our series using an univariate analysis, but these factors dropped out after multivariate adjustment. This might be due to the small number of events recorded in the present cohort. Our patients were younger (mean, 65 years; range, 35-87 years) compared to those in the previous study. With this age range, age appears not to be a predictive factor for UGIB.

The incidence of UGIB event in patients with CAD started on low-dose aspirin was low, and if occurred, was associated with good clinical outcome though most patients required hospitalisation. Nevertheless, with better understanding of the pathogenesis of the former and, in particular, its relation to *H pylori* status, our group has reported a possible method for the prevention of recurrence of peptic ulcer complication from long-term low-dose aspirin use[12]. On the other hand, our study showed that certain high-risk group of patients, especially those with history of peptic ulceration who tend to have early UGIB, should be target for primary prevention of peptic ulceration development and continued surveillance needed as well for these patients while on low-dose aspirin.

The small number of patients in our cohort that were on regular NSAID (1.1%) might not be able to show its deleterious effect on patients taking low-dose aspirin. Likewise, there was insufficient number of patients taking ulcer-protecting agents 21% to show a statistically significant benefit for patients taking these medications for ulcer prevention. However, the low usage of NSAID and ulcer-protecting drugs allowed us to determine the natural history of UGIB secondary to low-dose aspirin in this patient cohort, in which future therapeutic strategy can be planned. Finally, we failed to obtain the *H pylori* status for all patients, Regardless of whether they had or had not had an event, from which the value of that might be much more elicited. In addition, serological assay for *H pylori* infection was not universally available at time of our study. Nevertheless, local study reported that 55% of the population was infected with *H pylori* though symptoms and clinical disease developed in only a minority of infected individuals during their lifetime[13].

In summary, the incidence of UGIB in patients with CAD on low-dose aspirin remains low at 1.5% per year. Patients with hypertension, history of peptic ulcer problem, low body fat content (high lean body mass) and those that had received tertiary education were at risk of having an UGIB event. There exists bimodal presentation of the UGIB event with the early bleeders presenting in the first few months of starting treatment and the late bleeders after at least one year of therapy. Patients who had an event were likely to be hospitalized and required blood transfusion.

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RAPID COMMUNICATION

Relationship between IFN-γ gene polymorphism and susceptibility to intrauterine HBV infection

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Abstract

AIM: To explore the susceptibility of children to intrauterine HBV infection by studying the relationship between IFN-γ gene polymorphism, including IFN-γ+874A/T single nucleotide polymorphism(SNP) and CA repeat microsatellite polymorphism and intrauterine HBV infection.

METHODS: A TaqMan fluorescence polymerase chain reaction in the IFN-γ+874A/T single nucleotide polymorphism was tested in the intrauterine HBV infection group(group I) and the normal immune children group(group II). Capillary electrophoresis was performed in the above two groups to assay the IFN-γ CA repeat microsatellite polymorphism.

RESULTS: Frequencies of AA, AT and TT genotypes were 67.4%, 19.6% and 13.0% in the intrauterine HBV infection group, and 45.2%, 30.1% and 24.7% in the normal immune children group, respectively. A significant difference was found in the frequency distribution of IFN-γ+874 genotype between the two groups (χ^2 = 5.102, P = 0.02389). In the intrauterine HBV infection group the AA genotype was more common than in the normal immune group. Frequency of IFN-γ+874A allele was 77.17% in the intrauterine HBV infection group, and 60.27% in the normal immune children group. In the intrauterine HBV infection group the IFN-γ+874A allele was more common than in normal immune group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 7.238, P = 0.02389, OR = 2.228, 95% CI = 1.244-3.992). $(CA_{12})^+/(CA_{12})^+$ of IFN-γ CA microsatellite polymorphism was 11.90% in the intrauterine HBV infection group and 26.47% in the normal immune children group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 5.64, P = 0.0176). Frequency of IFN-γ CA repeat was 25% in the intrauterine HBV infection group and 43.38% in the normal immune children group. The frequency of IFN-γ CA repeat was less in the intrauterine HBV infection group than in normal immune group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 7.548, P = 0.0060).

CONCLUSION: There is a relationship between IFN-γ+874A/T SNP and intrauterine HBV infection as well as between IFN-γ CA microsatellite polymorphism and intrauterine HBV infection. IFN-γ gene polymorphism might be important in determining individual's susceptibility to intrauterine HBV infection.

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Key words: Interferon-γ; Gene polymorphism; Hepatitis B virus; Intrauterine

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INTRODUCTION

IFN-γ+874A/T SNP within the first intron can increase or decrease the binding of a particular transcription factor and ultimately the export of cytokine product[1-2]. The registered DNA sequences of the human IFN-γ gene deposited in Genbank show the presence of a CA repeat element in the first intron, 875 bp downstream from the start of the first exon. There are seven CA repeats of variable lengths In a consecutive study, 12 CA repeats are confirmed its role as a marker of high IFN-γ production[2]. We have reported in a previous study that higher IFN-γ secretion than spontaneous secretion could be found in the intrauterine HBV infection group and the normal immune children group with HBsAg stimulation, but the increase in the intrauterine HBV infection group is strikingly lower than that in the normal immune children group[3]. IFN-γ production is positively associated with IFN-γmRNA expression[3]. In the present study, we studied the IFN-γ+874 SNP and CA repeat microsatellite polymorphism in the intrauterine HBV infection group and the normal immune children group in order to explore the association between IFN-γ gene polymorphisms and susceptibility to intrauterine HBV infection.

MATERIALS AND METHODS

Subjects

The subjects were selected from outpatients who were in our hepatitis B (HB) vaccine follow up clinics. These subjects whose mothers were HBV carriers were inoculated with HB vaccine or HB vaccine plus hepatitis B immunoglobulin (HBIg) and followed up for serum alanine transaminase (ALT) and HBV marker. Intrauterine HBV-infected children were defined based on their positive HBsAg and/or HBV-DNA at birth and lasting for six months (group I). Normal immune children were defined based on their negative HBV marker after birth and high HBsAb titers (group II). ALT of all subjects was normal.

TaqMan fluorescence polymerase chain reaction

Genomic DNA was extracted from peripheral blood using the kits supplied by Sangon Bioengineering Company (Shanghai, China). Polymorphisms at +874 were identified using the ABI Prism 7700 fluorescent automatic sequencer. PCR products were obtained after amplification in a 10 μL volume containing 1×Taqman buffer A, 200 μmmol/L(each) dATP, dCTP, dGTP, 400 μmmol/LdUTP, 3.5 mmol/L $MgCl_2$, 300 μmmol/Lβ-actin forward primer, 300 μmmol/L β-actin reverse primer, 200 μmmol/Lβ-actin probe, AmpliTaq Gold, 0.025 U/μL DNA polymerase, 0.01 U/μL AmpErase UNG, 900 nmmol/L(each) forward & reverse primers, 250 nmol/L TaqMan MGB probe, 20ng DNA template. The forward primer sequence is 5'-ACA TTC CAC AAT TGA TTT TAT TCT TAC AAC A-3'. The reverse primer sequence is 5'-ACG AGC TTT AAA AGA TAG TTC CAA ACA-3'. The TaqMan MGB probe sequences are as follows: Fam: AAA TCA AAT CtC ACA CAC ACA C; Vic: AAA TCA AAT CaC ACA CAC ACA C. Forty-five cycles of PCR amplification were performed at 50 ℃ for 2 min, at 95 ℃ for 10 min, at 95 ℃ for 30 s and at 60 ℃ for 30 s.

Capillary electrophoresis

DNA was amplified in a 7.5 μL reaction volume containing 0.75 μL of 10 × buffer, 0.75 μL 25 mmol/L $MgCl_2$, 0.75 μL 2.5 μmmol/LdNTP, 2.5 U/μL Taq Gold, 0.06 μL DNA polymerase, 1 μL (each) forward & reverse primers, 2 μL DNA template, 1.19 μL ddH_2O. The forward primer sequence is CTT CGT TGC TCA CTG GGA TT-6-FAM. The reverse primer sequence is GCA AAG CCA CCC CAC TAT AA. The microsatellite region in the first intron of the IFN-γ gene was amplified on a PTC-100 thermal cycler. Following an initial denaturation (12 min at 95 ℃), samples were subjected to 10 cycles of PCR amplification, for each cycle, the sample was amplified at 94 ℃ for 30 s, at 53 ℃ for 30 s, at 75 ℃ for 45 s, followed by 30 cycles at 89 ℃ for 30 s, at 53 ℃ for 30 s, at 75 ℃ for 45 s, and a final extension at 4 ℃. The IFN-γ CA repeat microsatellite polymorphisms were monitored by ABI PRISM 3700 capillary electrophoresis automatic analysis.

Statistical analysis

IFN-γ+874A/T allele frequencies and IFN-γ CA microsatellite frequencies were evaluated by gene count. Allelic frequency was calculated with the following equation: allelic frequency = $n/2N$ (n represents the number of alleles, and N represents the total number of subjects). Allelic frequencies were compared using the chi-square test. A P value less than 0.05 was considered statistically significant. The strength of an association was expressed as odds ratio (OR) with 95% confidence interval. STATA 6.0 software was used to determine the statistical significance.

Table 1 Clinical characteristics of patients and controls

Subjects	*n*	Sex		HBV marker of mother		HBIg injection in pregnancy	
		Male	Female	$HBsAg^+$	$HBsAg^+$ and $HBeAg^+$	Yes	No
Group I	46	26	20	15	31	10	30
Group II	73	41	32	25	48	16	47
χ^2		0.0015		0.0339		0.0020	
P		0.9695		0.8539		0.9640	

Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

Table 2 Frequencies of IFN-γ+874 genotype in patients and controls

Subjects	*n*	Number of subjects and percentage of IFN-γ+874 genotype (%)		
		AA	AT	TT
Group I	46	31 (67.4)	9 (19.6)	6 (13.0)
Group II	73	33 (45.2)	22 (30.1)	18 (24.7)
χ^2		5.102		
P		0.02389		

Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

RESULTS

General clinical characteristics

No significant difference was found in sex, HBeAg positivity of mother and HBIg injection to pregnant women between the two groups (Table 1).

IFN-γ+874A/T SNP

The distribution of IFN-γ+874A/T allele frequencies in two groups was in accordance with Hardy-Weinberg hereditary equilibrium law. Frequencies of AA, AT and TT genotypes were 67.4%, 19.6% and 13.0% in the intrauterine HBV infection group and 45.2%, 30.1% and 24.7% in the normal immune children group, respectively. A significant difference was found in the frequency distribution of IFN-γ+874 genotype between the two groups ($\chi^2 = 5.102$, $P = 0.02389$). In the intrauterine HBV infection group, the AA genotype was more common than in normal immune group (Table 2). Frequency of IFN-γ+874A allele was 77.17% in the intrauterine HBV infection group and 60.27% in the normal immune children group. In the intrauterine HBV infection group, the IFN-γ+874A allele was more common than in normal immune group. A significant difference was found in the frequency distribution between the two groups ($\chi^2 = 7.238$, $P = 0.02389$, OR = 2.228, 95%CI1.244-3.992)(Table 3).

Table 3 +874T and +874A IFN-γ allele frequencies in patients and controls

	Allele (*n*)	IFN-γ+874A/T allele frequencies (%)		OR (95% confidence interval)	χ^2	*P*
		+874A	+874T			
Group I	46 (92)	71 (77.17)	21 (22.83)	3.228 (1.244-3.992)	7.238	0.0071
Group II	73 (146)	88 (60.27)	58 (39.73)			

Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

Table 4 IFN-γCA microsatellite morphisms in patients and controls

	n	Number and percentage of genotype of IFN-γCA (%)		
		$(CA_{12})+/(CA_{12})^+$	$(CA_{12})+/(CA_{12})^-$	$(CA_{12})^-/(CA_{12})^-$
Group I	42	5 (11.90)	11 (26.19)	26 (61.91)
Group II	68	18 (26.47)	23 (33.82)	27 (39.71)
χ^2			5.640	
P			0.0176	

Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

IFN-γ CA microsatellite polymorphism

$(CA_{12})^+/(CA_{12})^+$ of IFN-γ CA microsatellite polymorphism was 11.90% in the intrauterine HBV infection group and 26.47% in the normal immune children group. A significant difference was found in the frequency distribution between the two groups ($\chi^2 = 5.64$, $P = 0.0176$)(Table 4). Frequency of IFN-γ CA repeat was 25% in the intrauterine HBV infection group and 43.38% in the normal immune children group. In the intrauterine HBV infection group, the frequency of IFN-γ CA repeat was less than in normal immune group. A significant difference was found in the frequency distribution between the two groups ($\chi^2 = 7.548$, $P = 0.0060$)(Table 5, Figure 1).

Table 5 IFN-γ CA repeat times in patients and control

IFN-γ CA repeat	Group 1 (84) % (*n*)	Group II (136) % (*n*)	χ^2	*P*
11	1.19 (1)	0.74 (1)	0.119	0.7302
12	25 (21)	43.38 (59)	7.548	0.0060[1]
13	13.10 (11)	8.82 (12)	1.008	0.3155
14	40.48 (34)	33.82 (46)	0.989	0.3201
15	13.10 (11)	7.35 (10)	1.974	0.1600
16	4.76 (4)	3.68 (5)	0.155	0.6936
17	2.38 (2)	2.21 (3)	0.007	0.9327

[1] Significant difference between patients and controls.
Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

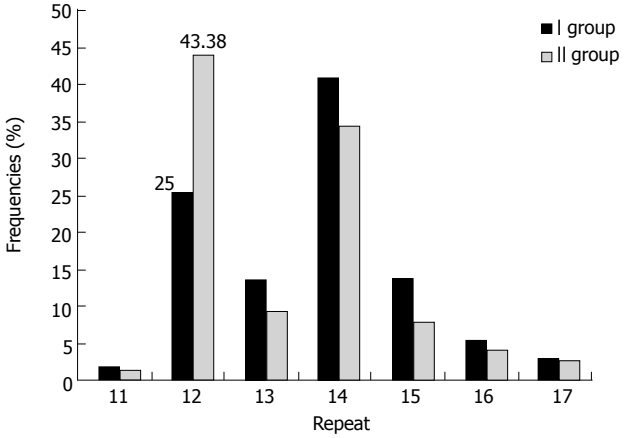


Figure 1 IFN-γ CA microsatellite polymorphisms in patients and controls. Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

DISCUSSION

The clinical features of HBV infection depend on the activities of the host immune response and the virus. The cell-mediated immune response to HBV-encoded antigen is responsible both for viral clearance and for disease pathogenesis during this infection. T-helper lymphocyte response mediated through secretion of IFN-γ plays an important role in inducing viral clearance[4]. During the early aspecific phase of host defence, production of IFN-γ by natural killer(NK) cells plays an important role in bringing about acute inflammation. In the subsequent antigen-specific phase of the immune response, IFN-γ acts as a regulator of antigen presentation and proliferation as well as differentiation of lymphocytes. High-level production of IFN-γ during this phase plays an important role in viral clearance[5-6].

Cytokine production is genetically controlled. The cytokine genes SNPs in cis-acting regions can alter transcriptional activity and are associated with the production of cytokine[7]. Genetic susceptibility to diseases is likely influenced by common DNA variants in the form of SNPs[8-9]. Pravica *et al*[2] described a variable length CA repeat sequence in the first intron of the human IFN-γ gene and showed that 12 CA repeat is associated with high *in vitro* IFN-γ production. In a further study, a SNP T to A at the end of the CA repeat region in the first intron of the human IFN-γ gene(+874A/T) was described, showing an absolute correlation between the T allele and the high IFN-γ microsatellite polymorphism. The +874T allele is associated with the high production of genotype and the +874A allele is associated with the low production of genotype[10]. *In vitro* production of IFN-γ has a significant correlation with the CA microsatellite polymorphism. Twelve CA repeats have been shown to have a higher expression of IFN-γ[11-12]. Japanese researchers[13] have reported the frequencies of 13 CA repeats are significantly greater in patients with IgA nephropathy than in the healthy control group (43% versus 23%, $P<0.05$). High production of genotype (12 CA repeats) for IFN-γ may have an influence on acute rejection of kidney transplants[14]. The frequency of the IFN-γ12 CA repeats is significantly greater in WHO class V lupus nephritis patients than in WHO class IV patients[15]. Susceptibility to and severity of rheumatoid arthritis are related to a microsatellite polymorphism

within the first intron of the IFN-γ gene[16]. There is a highly significant increase in the low-production of IFN-γ genotype(13 CA repeats) in patients with type 1 diabetes compared with normal healthy controls[17]. This result suggests that polymorphisms of the IFN-γ gene may modify the function of this proinflammatory mediator and the response to pancreatic islet βcells. This +874A/T polymorphism coincides with a putative NF-Kappa B binding site, which might have functional consequences for transcription of human IFN-γ gene. Therefore, the T to A polymorphism might directly influence the level of IFN-γ production associated with the CA microsatellite polymorphism[18]. Patients with tuberculosis have a lower frequency of +874TT genotype than the controls, suggesting that genetically determined variability in IFN-γ and expression might be important for the development of tuberculosis[19]. A significant correlation has been detected between the presence of high-expression polymorphisms of the IFN-γ genes and bronchiolitis obliteration syndrome(BOS) after lung transplantation($P = 0.039$). +874TT of the IFN-γ gene significantly increases the risk of BOS after lung transplantation[20]. There is no report on the relationship between IFN-γ gene polymorphism and susceptibility to intrauterine HBV infection.

In this study, the frequency of the +874AA genotype of the IFN-γ was greater in the intrauterine HBV infection group than in normal immune group, the $(CA_{12})^+/(CA_{12})^+$ of IFN-γ CA microsatellite polymorphism was 11.90% in the intrauterine HBV infection group and 26.47% in the normal immune children group. A significant difference was found in the frequency distribution between the two groups($\chi^2 = 5.640$, $P = 0.0176$). The frequency of IFN-γ CA repeat was less in the intrauterine HBV infection group than in the normal immune group. A significant difference was found in the frequency distribution between the two groups($\chi^2 = 7.548$, $P = 0.0060$). The findings suggest that the presence of low-expression polymorphism at +874 of the IFN-γ gene significantly increases susceptibility to intrauterine HBV infection. IFN-γ gene polymorphism might be important in determining an individual's susceptibility to intrauterine HBV infection and might result in viral persistence.

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RAPID COMMUNICATION

Diverticulitis in the young patient-Is it different ?

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Abstract

AIM: To prospectively study the incidence and the natural history of acute diverticulitis in young patients.

METHODS: A total of 207 patients hospitalized at our hospital between January 2000 to February 2005 with the diagnosis of acute diverticulitis were included. Their demographic characteristics, medical history, physical, radiographic and endoscopic findings as well as therapy were recorded. Patients were followed every 6 mo for the first year and later annually.

RESULTS: The mean patients' age was 61 (range 27-92) years. Twenty- five patients (12%) were younger than 45 years. Acute diverticulitis was significantly more prevalent among male in the young age group as compared to the older age group (19/25, 76% *vs* 61/182, 33%, respectively, $P = 0.0001$). Complications occurred more often in the young age group; 32% *vs* 13%, ($P = 0.002$). During follow-up, 6 patients (28%) remained asymptomatic in the young age group as compared to 87 patients (55%) in the older age group ($P = 0.024$). As a result, sigmoidectomies were performed twice as often in the young age group (38% *vs* 13%, $P = 0.002$).

CONCLUSION: Diverticulitis in young patients has a male predominance, a more aggressive course with a higher rate of complications and a higher recurrence rate. An earlier surgical approach might be considered in young patients with acute diverticulitis.

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Key words: Diverticulitis; Young patient; Complications; Sigmoidectomy

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INTRODUCTION

Diverticular disease of the colon is a common disease in Western countries. The development of colonic diverticula is associated with a low fiber diet, which is common in the developed countries[1,2]. The incidence of diverticular disease rises with age, and by the age of 85 about 65% of the population would have diverticuli[3,4]. It is much less frequent in the younger age group and less than 10% of the population under the age of 40 years would have diverticuli[5] . Most of the patients will remain asymptomatic, and only 10%-25% will become inflamed and evolve into diverticulitis[4,6-9]. The clinical presentation of acute diverticulitis varies widely from mild inflammation to full-blown perforation and peritonitis. In most patients however, the disease is mild and responds to antibiotic therapy[10] .

In recent years, there has been conflicting information regarding the incidence and natural history of diverticulitis in young patients. While earlier studies found the incidence to range between 2%-7%[11-15] , more recent studies reported a higher incidence ranging between 18%-34%[16,17]. The natural course and outcome of diverticulitis in the young patient has also been a matter of debate. While earlier studies reported a more severe course with a higher rate of complications in young patients with diverticulitis[1,2,11-14,18], recent reports suggested a milder course, not different from that in the general population[10,16]. All those studies, however, were retrospective. We performed a prospective study in which all patients with acute diverticulitis were enrolled and followed for a period of up to 5 years. We compared the natural history and the course of acute diverticulitis in the young age group to that of the older population.

MATERIALS AND METHODS

Patients

All patients with acute diverticulitis who were hospitalized at the Chaim Sheba Medical Center between January 2000 and February 2005 were enrolled. In fact, our patients' population included all patients who were diagnosed with acute diverticulitis in the emergency room, since the general policy of our institution is to hospitalize these patients regardless the severity of the disease.

Methods

The diagnosis of acute diverticulitis was based on lower

abdominal pain (usually on the left side), fever and leukocytosis. All patients underwent abdominal CT and only those with characteristic findings on CT were included. CT criteria for acute diverticulitis included the presence of colonic diverticuli with thickening of the colonic wall at the site of the diverticuli and peri-colonic fat infiltration. In some of the patients extra colonic air or fluid was seen as well. Patients with a lesion suspicious of colonic cancer on CT scan were excluded. Demographic characteristics of all patients and their medical history, concomitant use of medications, physical, laboratory, radiographic and endoscopic findings and their therapy were recorded.

Patients were followed up during their hospitalization and later by regular phone calls every 6 mo for the first year and then annually. Follow-up was conducted by a specialized study coordinator who was unaware of the patients' course of disease. At their follow-up visits or phone calls, patients were asked about the occurrence of attacks of lower abdominal pain with or without fever, hospitalizations and operations. Based on their reports, patients were classified into 4 major categories: patients who had undergone sigmoidectomy following their discharge, patients who had another episode of acute diverticulitis which required hospitalization and antibiotic therapy, patients who had LLQ abdominal pain for more than twice a week and for at least one hour's duration and patients who had not experienced symptoms suggestive of diverticulitis.

Data was analyzed for the whole group of patients and then for the two subgroups based on their age (older than and younger than 45 years). The age of 45 years was chosen as the average between the different ages used in the literature to define the young age group with acute diverticulitis (40 *vs* 50 years).

Statistical analysis

Patients' demography and other characteristics were analyzed by the Student's *t*-test for continuous variables and χ^2- test (with Yates correction) or Fisher's exact test for categorical variables. Statistical analyses were conducted by using Statsoft software (Tulsa, OK, USA).

RESULTS

Two-hundred and seven patients with acute diverticulitis were hospitalized between January 2000 and February 2005. Eighty were males and 127 were females. Their age ranged between 27-92 (mean 62 ± 12) years. Twenty-five patients (12%) were younger than 45 years. Their demography and co-morbidities are shown in Table 1. There was a striking male predominance in the young age group with a male: female ratio of 3:1. The older age group showed a female predominance with a female: male ratio of 2:1. As expected, the associated co-morbidities were far more common in the older age group, though only hypertension and ischemic heart disease reached statistical significance. The younger age group had almost no co-morbidities. However, twice as many abdominal operations had been performed in the young age group; 16% appendectomy and 12% inguinal hernioplasty as compared to 8.8% and 6%, respectively.

Table 1 Demographic data and co-morbidities of study subjects

Age (yr)	< 45 *n* = 25 (%)	>45 *n* = 182 (%)	*P* value
Gender (M/F)	19/6	61/121	0.0001
Co-morbidities			
Hypertension	1(4)	57 (31)	0.004
Hypothyroidism	0(0)	19 (10)	0.185
COPD	0(0)	10 (5.5)	0.481
Diabetes mellitus	0(0)	19 (10)	0185
Ischemic heart disease	0(0)	36 (20)	0.030
Hyperlipidemia	0(0)	17 (9)	0.227
Cholelithiasis	1(4)	11 (6)	0.682
Post appendectomy	4(16)	16 (8.8)	0.253
Post inguinal hernioplasty	3(12)	11 (6)	0.266
Malignancy	0(0)	7 (3)	0.684
Crohn's disease	1(4)	1 (0.5)	0.100
Chronic renal failure	0(0)	4 (2.2)	0.979

COPD: chronic obstructive pulmonary disease.

Table 2 Patients' complications during hospitalization

Age (yr)	< 45 *n* = 25 (%)	>45 *n* = 182 (%)	*P* value
Peri-diverticular air on CT	5 (20)	15 (8)	0.063
Abdominal abscess	2 (8)	4 (2)	0.150
Free perforation	1 (4)	5 (3)	0.540
Death	0 (0)	2 (1)	0.598
Total complication rate	8 (32)	24 (13)	0.030

Clinical course during hospitalization

All patients were followed during hospitalization and their clinical outcome was recorded. One-hundred and seventy-five patients (85%) had a mild course with no complications. The other 32 patients had a more severe and complicated course (Table 2). The most common complication was peri-diverticular air on CT which resolved in all patients by keeping patients fasting and on IV fluids and antibiotics. Six patients (2.8%) developed an abdominal abscess and 3 required CT guided percutaneous drainage. The abscesses resolved in all patients with no need for further intervention. There were 6 patients (2.8%) who developed free perforation and all were operated on. Two patients (0.1%) died during hospitalization, one from sepsis and another from multi-organ failure. Both were older than 80 years and had several co-morbidities. Their deaths were related to the diverticulitis.

The majority of the complications were more common in the young age group (Table 2). Abdominal abscess (8% in the young group *vs* 2% in the old group, $P = 0.155$) and peri-diverticular air on CT (20% in the young *vs* 8% in the old, $P = 0.063$) were the most common. The presence

Tables 3 Patients' follow-up after discharge

Age (yr)	<45 *n* = 21(%)	>45 *n* = 159 (%)	*P* value
Operations following discharge	8 (38)	20 (13)	0.002
Recurrent diverticulitis (not operated)	2 (10)	20 (13)	0.688
Abdominal pain[1]	5 (24)	27 (17)	0.442
No further symptoms	6 (28)	87 (55)	0.024

[1]Abdominal pain- pain more than 2 times per week that lasts for at least one hour.

of free perforation, however, was almost equal in both groups; 4% in the young and 3% in the old age group. The overall complication rate was higher in the young age group ($P = 0.03$).

Follow-up

Patients were contacted by telephone every 6 mo for the first year and yearly thereafter. The range of follow up was between 6 and 60 mo, mean: 30 ± 14 mo. Four patients in the young age group (16%) and 23 patients in the old age group (13%) were lost to follow up. Three of the patients in the old age group (2%) died during the follow-up period, all of whom were older than 75 years and their deaths were not related to the diverticular disease.

Data on patients in whom complete follow up was achieved is shown in Table 3. Sigmoidectomy was significantly more often performed in the young age group (38% *vs* 13%, $P = 0.002$). Most of the patients in the older age group, unlike the younger one, were asymptomatic during their follow up (55% *vs* 28%, respectively, $P = 0.024$).

DISCUSSION

Our prospective study showed that patients younger than 45 years comprised 12% of all patients who presented to our hospital with acute diverticulitis. The younger age group was male predominant and tended to have a more severe course with a higher complication rate, mainly peri-diverticular air and intra-abdominal abscess. Their recurrence rate during follow-up was higher and as a result their rate of sigmoidectomy was higher also. In 1986, Freischlag *et al*[13] found that in young patients, the first onset of diverticulitis was often severe and required urgent operation. Other retrospective studies supported this impression[1,2,18] and described a high recurrence rate and a high complication rate in their young patients with diverticulitis[19,20]. It was therefore recommended that an elective operation should be performed following the first episode of diverticulitis[17,19,21]. This policy was even further supported by Chautems *et al* who followed patients with acute diverticulitis under the age of 50 years for nearly 10 years. They found that in the young age group patients experienced a more aggressive disease[22]. However, other studies published in the last decade, failed to support this finding[10,16,23-26]. These studies reported a course of disease in young patients not different from the older age group and with a similar rate of complications. The conclusion of those recent studies was that elective surgery after one episode of acute diverticulitis in young patients is not advocated and such patients should be managed in a similar way to the older ones[25,26]. The explanation for the difference between studies that either support or reject a severe course in young patients is not clear. A possible explanation might be the difference in the study populations. Studies that found a similar course in both age groups had in their young age group a high proportion of Latino originated patients[10,16,23]. Young Latinos were reported to have an increased tendency to develop acute diverticulitis[27]. On the other hand, studies that described a severe course in young patients with acute diverticulitis did not include the Latino originated population. In fact, some of the studies were performed in Europe where Latino origin is quite rare[19,20,22]. Our study population also did not have patients of Latino origin. Thus, we speculate that young Latino patients with acute diverticulitis have a more benign course similar to that of older patients. The reason for this is not clear.

As a group, our young patients were more prone to recurrent symptoms. Our data is consistent with earlier studies that reported a higher recurrence rate of diverticulitis among young patients[2,20].The total complication rate was significantly higher in the young age group. None of the specific complications (free perforation, abdominal abscess or peri-diverticular air) reached statistical significance, probably because of the relative small number of patients in the young age group. Death occurred only in the older age group probably as a result of their higher co-morbidity rates.

In our study, the young patients with acute diverticulitis underwent significantly more sigmoidectomies as compared to our older patients. All those who underwent a sigmoidectomy had recurrent episodes of diverticulitis. The high rate of sigmoidectomies in the young age group was, therefore, the result of a more severe course of the disease in patients who were otherwise in good general condition.

Of interest is that the young age group underwent twice as many appendectomies and inguinal hernioplasties as compared to the older age group. Acute appendicitis in the young age group was even more prevalent than the life time risk for acute appendicitis in the general population (estimated 8.6% in men and 6.7% in women)[28]. The significance, if at all, of this observation is unclear. Misdiagnosis of previous attacks of acute diverticulitis is a possible explanation.

In conclusion, our prospective, long-term follow-up study shows that acute diverticulitis in general is a mild disease with a low complication rate. In young patients there is male predominance and the disease tends to be more aggressive with a high recurrence rate. An earlier surgical approach might be considered in young patients with acute diverticulitis.

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RAPID COMMUNICATION

Effects of ischemic preconditioning on cyclinD$_1$ expression during early ischemic reperfusion in rats

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Abstract

AIM: To observe the effect of ischemic preconditioning on cyclinD$_1$ expression in rat liver cells during early ischemic reperfusion.

METHODS: Fifty-four SD rats were randomly divided into ischemic preconditioning group (IP), ischemia/reperfusion group (IR) and sham operation group (SO). The IP and IR groups were further divided into four sub-groups (n = 6). Sham operation group (SO) served as the control group (n = 6). A model of partial liver ischemia/reperfusion was used, in which rats were subjected to liver ischemia for 60 min prior to reperfusion. The animals in the IP group underwent ischemic preconditioning twice for 5 min each time prior to the ischemia/reperfusion challenge. After 0, 1, 2, and 4 h of reperfusion, serum and liver tissue in each group were collected to detect the level of serum ALT, liver histopathology and expression of cyclinD$_1$ mRNA and protein. Flow cytometry was used to detect cell cycle as the quantity indicator of cell regeneration.

RESULTS: Compared with IR group, IP group showed a significantly lower ALT level in 1 h to 4 h sub-groups (P < 0.05). Proliferation index(PI) indicated by the S-phase and G$_2$/M-phase ratio [(S+G$_2$/M)/(G$_0$/G$_1$+S+G$_2$/M)] was significantly increased in IP group at 0 and 1 h (26.44 ± 7.60% *vs* 18.56 ± 6.40%,41.87 ± 7.27% *vs* 20.25 ± 6.70%, P < 0.05). Meanwhile, cyclinD$_1$ protein expression could be detected in IP group. But in IR group, cyclinD$_1$ protein expression occurred 2 h after reperfusion. The expression of cyclinD$_1$ mRNA increased significantly in IP group at 0 and 1 h (0.568 ± 0.112 *vs* 0.274 ± 0.069, 0.762 ± 0.164 *vs* 0.348 ± 0.093, P < 0.05).

CONCLUSION: Ischemic preconditioning can protect liver cells against ischemia/reperfusion injury, which may be related to cell proliferation and expression of cyclinD$_1$ during early ischemic reperfusion.

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Key words: Liver; Ischemic preconditioning; CyclinD$_1$; Proliferation

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INTRODUCTION

Hepatic ischemia/reperfusion (IR) injury is an important clinical problem occurring in hepatic resection or liver transplantation. Ischemic preconditioning (IP) has been proven to be an effective method protecting the liver against ischemia/reperfusion injury[1-3]. Many mechanisms are associated with this phenomenon[4-8], but some are still unclear. Previous studies showed that the analogous conditioning phenomenon induced by carbon tetrachloride (CCl_4) is related to hepatocellular proliferation[9]. Whether IP promotes hepatocellular regeneration remains unknown. Changes in cell proliferation can be determined by cell cycle analysis. S-phase and G$_2$/M-phase ratio is an indication the proliferation index (PI). Furthermore, cyclin and cyclin-dependent kinases (CDKs) are related to cell cycle regulation[10]. CyclinD$_1$ is an important cyclin, which controls liver cell proliferation[11]. This study was to assess the effects of IP on cell regeneration and expression of cyclinD$_1$ and to explore the possible mechanisms of IP protection.

MATERIALS AND METHODS

Experimental animals and grouping

Fifty-four male SD rats weighing 200-250 g, were randomly divided into ischemia group (IR group), ischemia preconditioning group (IP group) and sham operation group (SO). In IP group, each rat was subjected to 5 min

ischemia, followed by 5 min reperfusion, twice, prior to 1 h hepatic ischemia. Specimens were taken after 0, 1, 2, and 4 h of reperfusion, respectively (n = 6). Before the experiment, all rats were fasted for 12 h and housed in a 12 h dark/12 h light cycle.

Experimental animal model

A 70% partial liver ischemia/reperfusion model was established according to the method reported by Ohmori *et al*[12]. After anesthesia with ketamine (40 mg/kg), the belly of each rat was cut open from the center. The portal vein and hepatic artery supplying the left and median lateral hepatic lobes were dissected and clamped with a small artery clamp. After 1 h ischemia, blood flow was restored by unclamping the vessels. After 0, 1, 2, and 4 h of reperfusion, serum and liver tissue of each group were collected to assess the level of serum ALT, liver histopathology, expression of cyclinD$_1$ mRNA and protein. Liver tissue samples were homogenized and washed with PBS. The solution was filtered and centrifuged at 1000 r/min for 5 min. The cell pellet was resuspended in ice-cold ethanol to a final concentration of 80%. Flow cytometer was used to detect liver cell S-phase and G$_2$/M-phase ratio as the quantity indicator of cell regeneration. In IP group, each rat was subjected to 5 min ischemia followed by 5 min reperfusion, twice, prior to 1 h hepatic ischemia. The other procedure was the same as in IR group. In SO group, each rat was only cut open from the center without any other procedure.

Histological examination of liver

Liver tissue specimens were procured from the left lateral hepatic lobe, fixed with 10% neutral formaldehyde solution and embedded with paraffin. Sections were made and stained with H&E and observed under microscope.

Measurement of blood concentrations of ALT

Three mL of blood was withdrawn from the vena cava at certain time points, coagulated at room temperature, then centrifuged at 3000 r/min for 5 min for detecting the levels of alanine transaminase (ALT) with an automatic biochemistry analyzer (AU800, Olympus, Japan).

Detection of cell cycle

Single cell suspensions of hepatocytes were washed three times in PBS. RNase (1% w/v) was then added for 30 min at 37 ℃ and 50 mg/L PI (Sigma Company, Danmark) for 20 min. Samples were detected with a FACSORT flow cytometer (Becton Dickinson Company, USA), 10 000 cells were obtained by Cell Quest software and cell cycle was analyzed by Modfit software.

CyclinD$_1$ mRNA detection by reverse transcription-polymerase chain reaction (RT-PCR)

Primers synthesized according to the GenBank of Bioya Biotechnology Limited Company(Shanghai, China) included cyclinD1 sense primer (5'-TGGAGCCCCTGA AGAAGAG) cyclinD1 anti-sense primer (5'- AAGTGCGTTGTGCGGTAGC -3') amplified products (424bp), GAPDH sense primer (5'-GGCTGAGAATGGGAAGCTG-GTCAT-3') and GAPDH anti-sense primer (5'-CAGCCTTCTCCATGGTGGTGAA- GA-3) amplified products (152bp).

Total RNA was extracted from the frozen liver specimens with Trizol (GibcoBrl Company, USA). Reverse transcription synthesis of cDNA was carried out according to the instructions of SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen Company, USA). PCR was performed with a Gene Amp PCR System 2400 (PE Company, USA). The reaction system was composed of a cDNA template (1.0 μL), 2×buffer (12.5 μL), 5 mmol/L $MgCl_2$(2.5 μL), cyclin D1, GAPDH sense and anti-sense primer (0.5 μL respectively), SSRT polymerase (0.5 μL) and deionized water (6.5 μL). The reaction system was put into a PCR machine for amplification. Cyclin D1 and GAPDH were amplified for 35 cycles of PCR. The amplification conditions were at 55 ℃ for 30 min, at 94 ℃ for 2 min, at 94 ℃ for 30s, at 55 ℃ for 30 s, at 72 ℃ for 45 s, a final extension at 72 ℃ for 7min. The amplified products were preserved at 4 ℃. Ten μL of PCR products was electrophoresed on 1.5% agarose gel containing 5 μg/mL ethidium bromide (Sigma Company, USA). Marker was DL-2000 DNA marker. The results were detected by GeneSnap (SynGene, England) and analyzed by GeneTools (SynGene, England).

CyclinD$_1$ protein detection

Liver tissues were homogenized in 10 volumes of ice-cold buffer containing 50 mmol/L Tris–HCl (pH7.5), 150mmol/L NaCl, 1%Triton-X 100, 1 mmol/L EDTA,1mmol/L DTT,10μg/mL pepstatin,10μg/mL leupeptin, 10 μg/mL aprotinin, 1mM PMSF. The homogenates were centrifuged at 12 000 r/min for 15 min at 4 ℃. The supernatant was frozen and stored at -80 ℃ for further analysis. After measurement of the supernatant sample concentration, the sample was mixed with an equal volume buffer containing 0.1mol/LTris (pH 6.8), 100 mmol/L DTT, 2% SDS, 10% glycerin, 0.01% bromophenol blue and put in water bath to boil for 3-5 min. Fifty micrograms of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The stacking and resolving gels were composed of 4% and 10% polyacrylamide, respectively. After SDS-PAGE, the gels were electroblotted onto nitro-cellulose (NC) membranes at 100 mA for 2 h. The membranes were blocked for 1 h in blocking solution at room temperature, which contained 1 mL/L Tween 20 (PBS-T) and 50 g/L non-fat dry milk. Then they were immunolabeled with the primary antibody (1:500 rabbit anti-rat Santa Cruz Company, USA) in blocking solution for 1 h at room temperature and was washed three times with TBST for 15 min. The NC membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000 goat anti-rabbit Santa Cruz Company, USA) for 1 h at room temperature and washed three times with TBST for 15 min again. Color solution of diaminobenzidine (DAB) was prepared according to the manufacturer's directions (Maxim Company, China). After incubation with this color solution, the protein bands were visible on the NC membranes.

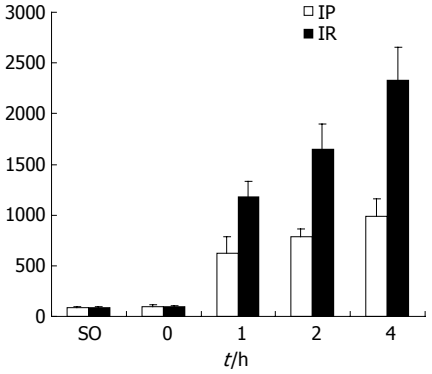


Figure 1 Serum ALT levels SO, IP and IR groups.

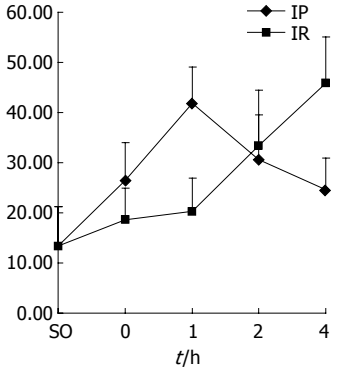


Figure 2 PI in SO, IP and IR groups.

Table 1 CyclinD1 mRNA relative activity in different groups (mean ± SD)

Group	IR	IP
SO	0.256 ± 0.042	0.256 ± 0.042
0 h	0.274 ± 0.069	0.568 ± 0.112[a,b]
1 h	0.348 ± 0.093	0.762 ± 0.164[a,b]
2 h	0.521 ± 0.157[b]	0.574 ± 0.172[b]
4 h	0.709 ± 0.178[b]	0.474 ± 0.144[a,b]

[a]$P < 0.05$ *vs* IR, [b]$P < 0.05$ *vs* SO.

Statistical analysis

The results were presented as mean ± SD. Multivariate analysis of variance and *t* test were performed by SPSS11.5 for window. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology

Swelling of hepatocytes and structural derangement were observed in IR group during the early period of reperfusion. Spot necrosis, hemorrhage and inflammatory cell infiltration could be seen in hepatic lobes or around central veins, with extended reperfusion time. Compared with IR group, IP group showed moderate hepatocyte swelling and less necrotic area.

ALT

The levels of serum ALT increased gradually as reperfusion time was extended in both groups. Compared with IR group, the level of serum ALT in IP group was significantly lower at all time points after reperfusion ($P < 0.05$) (Figure 1).

Hepatocellular proliferation

PI increased significantly at 0 and 1 h of reperfusion in IP group compared with IR group. There was a statistically significant difference ($P < 0.05$) (Figure 2).

Expression of cyclinD1 mRNA and protein

The RT-PCR results showed that cyclinD1 mRNA was expressed in SO, IR and IP groups. The expression of cyclinD1 mRNA in IP group increased at 0-1 h after reperfusion. Compared with IP group, the expression of cyclinD1 mRNA in IR group was significantly lower at 0-1 h after reperfusion ($P < 0.05$). No significant difference in the expression of cyclinD1 mRNA was found between SO and IR groups at 0-1 h after reperfusion ($P > 0.05$, Table 1, Figure 3A).

CyclinD1 protein was not detectable until 2 h after reperfusion in IR and SO groups. Whereas cyclinD1 protein was expressed in IP group soon after the reperfusion was begun (Figure 3B).

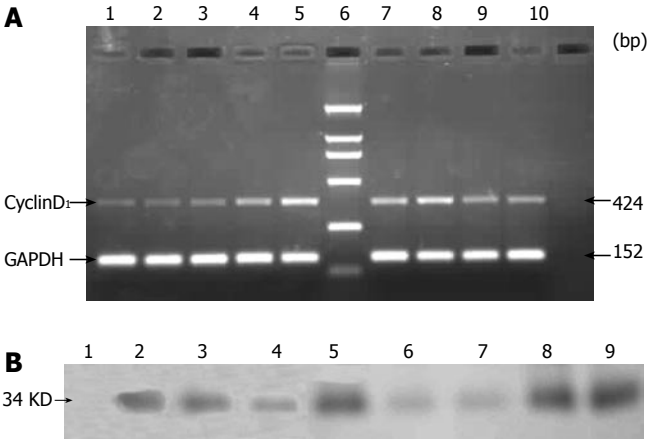


Figure 3 Expression of cyclin D1 mRNA (**A**) and cyclin D1 protein (**B**) at different time points in SO, IP and IR groups.

DISCUSSION

It is widely accepted that IP protects organs against IR injury. It has been reported that IR injury is related with many mechanisms, such as suppression of inflammatory reaction[13], improvement of local microcirculation[14], diminution of cell apoptosis and decreased production of local active oxygen-derived free radicals[15,16]. But some of the mechanisms remain uncertain. To probe into the mechanisms of IP protection may help to find an effective method against IR injury. In this study, we adopted the rat model of ischemic preconditioning against subsequent hepatic IR injury and confirmed that it could reduce the levels of serum ALT and the extent of liver necrosis.

Application of flow cytometry to cell cycle analysis makes it possible to analyze cell proliferation. Cell cycle can discriminate cells at the G_0/G_1-phase, S-phase and G_2/M-phase. It is considered that cells at the S-phase and G_2/M-phase can proliferate. S-phase and G_2/M-phase ratio could indicate the proliferation index (PI).The finding of the present work is that IP could promote liver cell proliferation during early ischemic reperfusion, which is possibly related to IP protection. Zhang *et al*[17] showed that proliferating liver cells can resist IR injury. The mechanisms of cellular proliferation involved in IP are still obscure, but

may be related to reactive oxygen species (ROS) and tumor necrosis facter-α (TNF-α).The liver underlying IP could generate ROS and TNF-α during early reperfusion[18,19]. ROS could stimulate cell mitosis and proliferation[20]. TNF-alpha is a known mitogen for hepatocytes and an important factor for liver cell proliferation. In the presence of anti-TGF-alpha neutralizing Abs, the mitogenic activity of TNF-alpha is abrogated[21].

Breakthrough in cell cycle regulation is the understanding of cyclin and cyclin-dependent kinase, which regulates cell cycle progression. Mitotic cellular division requires cells to leave the resting state (G_0/G_1) and proceed through the phase of DNA synthesis (S) and mitosis (G_2/M). There is a check point between G_0/G_1-phase and S-phase. CyclinD$_1$ is an important cyclin which can make cells pass through the check point and enter S-phase and G_2/M-phase. Higher expression of cyclinD$_1$ could promote cell proliferation in rat liver[11]. Our data showed that IP could stimulate cyclinD$_1$ mRNA and protein expression during the early reperfusion in IP group and not in IR group. The mechanisms may be related to the fact that IP activates the p38MAPK pathway[22], leading to activation of regulatory proteins and production of DNA synthetic proteins, such as cyclinD$_1$ and interleukin-6(IL-6)[23]. The p38MAPK pathway is an important signaling pathway involved in IP. Blockade of the p38MAPK pathway can lead to loss of protection of IP[24]. When the p38MAPK pathway is activated, the p38kinase transfers to cell nuclei and induces the production of cyclinD$_1$[25]. In our study, the cyclinD$_1$ protein was expressed in IR group 2 h after reperfusion, although cyclinD$_1$ mRNA was expressed during the early reperfusion. In SO group, cyclinD$_1$ mRNA was expressed but cyclinD$_1$ protein was not expressed. These data suggest that cyclinD$_1$ expression is posttranscriptionally regulated. Awad and Gruppuso[26] showed that a posttranscriptional mechanism regulating cyclin D1 content is involved in temporary hepatocyte growth arrest. They speculated that this posttranscriptional regulation may be downstream from the p38 mitogen-activated protein kinase pathway. The posttranscriptional regulation could keep adult hepatocytes in a quiescent state, but the mechanism is not completely clear.

In conclusion, IP could promote cyclinD$_1$ expression and lead to hepatocellular proliferation during early ischemic reperfusion, which is possibly one of the protective mechanisms against IR injury. However their underlying regulatory pathway and biological role remain unclear.

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RAPID COMMUNICATION

Lymph node micrometastasis and its correlation with MMP-2 expression in gastric carcinoma

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Abstract

AIM: To examine matrix metalloproteinase-2 (MMP-2) expression in gastric cancer tissues and to evaluate its relationship with lymph node micrometastasis.

METHODS: The authors studied 850 lymph nodes resected from 30 patients with gastric carcinoma who underwent gastrectomy with lymphadenectomy using reverse transcription polymerase chain reaction (RT-PCR) assay in addition to H-E staining. MMP-2 expression of the tumor tissues was detected by immunohistochemical technique (EliVision™ plus).

RESULTS: MMP-2 expression was positive in 21 (70%) cases and negative in 9 (30%) cases. No significant correlations were found between MMP-2 expression and other variables such as age, gender, tumor location, tumor diameter, Lauren classification and lymphatic invasion. In contrast, MMP-2 expression correlated significantly with depth of tumor infiltration (P = 0.022), lymph node metastasis (P = 0.030) and tumor differentiation (P = 0.043). Lymph node micrometastases were detected in 77 (12.5%) lymph nodes of 14 (46.7%) gastric carcinoma patients. MMP-2 expression was positive in 12 (85.7%) of the 14 patients with lymph node micrometastasis, and in 9 (56.3%) of the 16 patients without lymph node micrometastasis (P = 0.118).

CONCOUSION: Our results demonstrate that MMP-2 expression has significant correlation with tumor invasion, tumor differentiation and lymph node metastases. MMP-2 expression may be an important biological characteristics and significant prognostic parameter of gastric carcinoma. We also conclude that MMP-2 may participate in the development of lymph node micrometastasis of gastric carcinoma. Further investigations are needed to draw a conclusion.

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Key words: Gastric carcinoma; Lymph node micrometastasis; MMP-2; RT-PCR; Immunohistochemistry

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INTRODUCTION

Lymph node metastasis is the most important prognostic factor of gastric carcinoma. Many clinical studies demonstrate that the existence of lymph node micrometastasis is often overlooked by conventional histopathological examination (H-E staining)[1-10]. However, the mechanisms of lymph node micrometastasis remain unclear heretofore. MMP-2 has been reported to be associated with tumor invasion and lymph node metastasis of gastric carcinoma by mediating the degradation of extracellular matrix components[11-14]. However, the relationships between MMP-2 expression in cancer tissues and lymph node micrometastasis have not yet been explored. Therefore, the aim of this study was to investigate correlations between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma and possible relationships between lymph node micrometastasis and MMP-2 expression.

MATERIALS AND METHODS

Patients and specimens

A total of 850 lymph nodes resected from 30 patients with gastric carcinoma who underwent gastrectomy at the Department of Gastrointestinal and Pancreatic Surgery at Sun Yat-sen University of Medical Sciences were studied. There were 17 men and 13 women, ranging in age from 26 to 82 years, with a mean age of 56.8 years. None of these patients had received preoperative chemotherapy or radiotherapy. Total gastrectomy was performed in

16 patients, distal subtotal gastrectomy in 13 patients, and proximal subtotal gastrectomy in 1 patient. One patient underwent D1 lymphadenectomy, 22 patients underwent D2 lymphadenectomy, 4 patients underwent D3 lymphadenectomy, and 3 patients underwent palliative resection. According to the Lauren' criteria, 19 tumors were classified as diffusive type carcinomas, and 11 tumors were intestinal type carcinomas. Depth of tumor invasion and extent of lymph node metastasis were classified according to UICC TNM classification.

Half of each resected lymph node was fixed in 10% formalin and embedded in paraffin for routine histopathological examination. The other half was stored in 1ml RNA later (Sigma, USA) at 4 ℃ overnight, then transferred to a clean freezing tube and stored at -70 ℃ for RNA extraction. The resected primary tumors were also fixed in 10% formaldehyde and embedded in paraffin. Consecutive 4 μm sections were cut and stained with hematoxylin and eosin (H-E) staining and immunohistochenistry with anti-MMP-2 antibody.

RNA extraction

Lymph node samples were homogenized in 1 mL of Trizol Reagent (Invitrogen) per 50-100 mg of tissue using a power homogenizer. RNA extraction was carried out according to the protocol recommended by the manufacturer. Total RNA was dissolved in diethylpyrocarbonate-treated water and the volume and quality of the RNA then assessed by the ultraviolet spectrophotometer.

RT-PCR

Complementary DNA (cDNA) was synthesized and amplified from total RNA using the Access RT-PCR system (Promega). The primer sequences used for CK-20 detection were 5'-ggtcgcgactacagtgcatattaca-3' (sense) and 5' -cctcagcagccagtttagcattatc-3' (anti-sense)[15]. cDNA synthesis was monitored by beta-actin RT-PCR using the following primers: 5'- caaatgcttctaggcggact -3' (sense) and 5' - atgctatcacctcccctgtg -3' (anti-sense). The RT-PCR was performed in a 25 μL reaction mixture containing 11 μL nuclease-free water, 5 μL 5 × Reacton Buffer, 0.5 μL dNTP (10 mmol/L), 0.5 μL each of beta-actin primers (20 μmol/L), 1.25 μL each of CK-20 primers (10 μmol/L), 1 μL MgSO4 (25 mmol/L), 0.5 μL AMV reverse transcriptase (5 u/μL), 0.5 μL Tfl DNA polymerase (5 u/μL), and 3 μL RNA sample. The Access RT-PCR condition was set up as follows: 1 cycle at 48 ℃ for 45 min (reverse transcription), 1 cycle at 94 ℃ for 2 min (AMV RT inactivation), followed by 40 cycles at 94 ℃ for 30 s (denaturation) and at 62 ℃ for 1 min (annealing) and at 68 ℃ for 1.5 min (extension), followed by a final extension at 68 ℃ for 7 min. The resultant cDNA products of CK-20 and beta-actin were 121 base pairs and 381 base pairs, respectively. The RT-PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide.

In this report, "micrometastasis" in the regional lymph nodes was defined as metastasis that was detected only by the RT-PCR assay but not by routine H-E staining (Figure 1).

Immunohistochemistry

MMP-2 immunohistochemical staining was performed using EliVision™ plus IHC Kit (Maixin Biological, Fuzhou, China). After the sections were deparaffinized with xylene and dehydrated with ethanol, they were placed in 10 mmol/L citrate buffer and heated in a microwave at 700 W for 5 min for the retrieval of antigens in the specimens. Endogenous peroxidase activity was blocked by incubation of the slides in 3% hydrogen peroxide in absolute methanol at room temperature for 10 min. The sections were then incubated sequentially with 50 μL of mouse monoclonal antibody against MMP-2 (Maixin Biological, Fuzhou, China) overnight at 4 ℃, with 50 μL of polymer enhancer for 20 min, and 50 μL of polymerized HRP-anti mouse IgG for 30 min. The reaction products were visualized with diaminobenzidine (DAB Kit, Maixin Biological, Fuzhou, China), and sections were counterstained with hematoxylin, dehydrated, and evaluated under light microscope. Tris-buffered saline (TBS) solution was used instead of the primary antibody for negative controls.

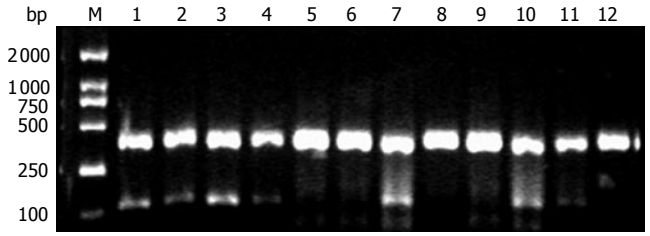


Figure1 Comparison of the results of RT-PCR and H-E staining. M: DL 2000 Marker; Lanes1-12: dissected lymph nodes. Lymph nodes of Lane 7, Lane 10, and Lane 11 were diagnosed lymph node micrometastasis.

For the purpose of data analysis, MMP-2 expression was graded according to the proportion of positive tumor cells. If more than 25% of the tumor cells were positively stained for MMP-2, the tumor was classified as positive MMP-2 expression. In contrast, if 25% or less of the tumor cells were positively stained, the tumor was classified as negative MMP-2 expression (Figure 2). The stained sections were observed independently by two pathologists who had no knowledge of the clinicopathological data.

Statistical analysis

Statistical analysis was performed by the Fisher's exact test to examine the association of MMP-2 expression of tumor tissues with the clinicopathologic characteristics of gastric carcinoma, and the relationship between MMP-2 expression of tumor tissues and lymph node micrometastasis was evaluated. Statistical significance was defined as $P < 0.05$.

RESULTS

Correlations between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma

MMP-2 expression was positive in 21 (70%) cases and negative in 9 (30%) cases. No significant correlations were found between MMP-2 expression and other variables such as age, gender, tumor location, tumor diameter, Lauren classification and lymphatic invasion. In contrast, MMP-2 expression correlated significantly with depth of tumor infiltration ($P = 0.022$), lymph node metastasis ($P = 0.03$) and tumor differentiation ($P = 0.043$) (Table 1).

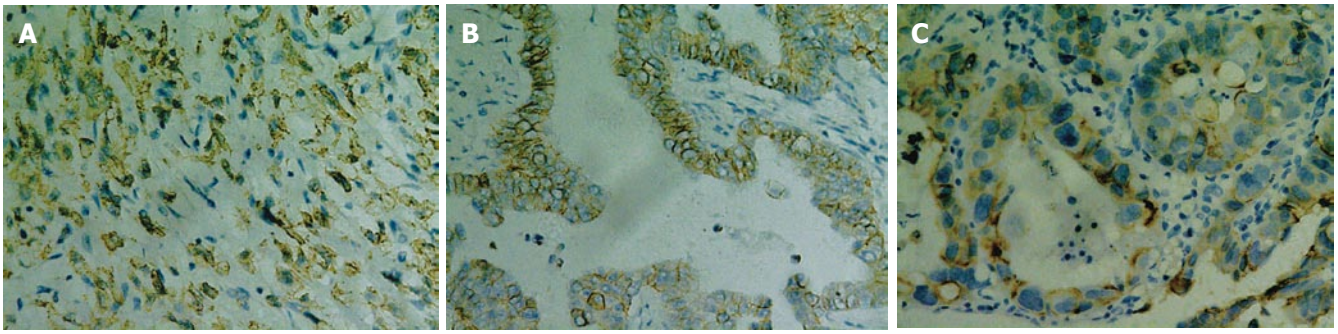


Figure 2 Expression of MMP-2 in gastric carcinoma. **A:** poorly-differentiated gastric carcinoma (× 400); **B:** moderately -differentiated gastric carcinoma (× 400); **C:** well-differentiated gastric carcinoma (× 400).

Table1 Correlation between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma

Variable	Patients (*n*)	MMP-2 expression		
		Negative (%)	Positive (%)	*P* value
Gender				
Male	17	6 (35.3)	11 (64.7)	
Female	13	3 (23.1)	10 (76.9)	*P* = 0.691
Age				
< 50 yr	10	1 (10)	9 (90)	
⩾ 50 yr	20	8 (40)	12 (60)	*P* = 0.204
Superficial diameter				
< 5 cm	17	7 (41.2)	10 (58.8)	
⩽ 5 cm	13	7 (15.4)	6 (84.6)	*P* = 0.229
Tumor location				
Upper/Middle third	17	5 (29.4)	12 (70.6)	
Lower third	13	4 (30.8)	9 (69.2)	*P* = 1.000
Histologic type				
Intestinal	11	5 (45.5)	6 (54.5)	
Diffuse	19	4 (21.1)	15 (78.9)	*P* = 0.225
Depth of invasion				
T1	5	4 (80)	1 (20)	
T2	16	4 (25)	12 (75)	
T3/T4	9	1 (11.1)	8 (88.9)	*P* = 0.022
Histologic differentiation				
Well	7	4 (57.1)	3 (42.9)	
Moderate	6	3 (50)	3 (50)	
Poorly	17	2 (11.8)	15 (88.2)	*P* = 0.043
Lymph node metastasis				
Positive	20	3 (15)	17 (85)	
Negative	10	6 (60)	4 (40)	*P* = 0.030
Lymphatic invasion				
Positive	18	3 (16.7)	15 (83.3)	
Negative	12	6 (50)	6 (50)	*P* = 0.102

Table 2 Correlation between MMP-2 expression of tumor tissues and lymph node micrometastasis

Micrometastasis	Patients (*n*)	MMP-2 expression		
		Negative (%)	Positive (%)	*P* value
Positive	4	2 (14.3)	12 (85.7)	
Negative	16	7 (43.7)	9 (56.3)	*P* = 0.118

Relationships between lymph node micrometastasis and MMP-2 expression

Lymph node micrometastases were detected in 77 (12.5%) lymph nodes of 14 (46.7%) gastric carcinoma patients. MMP-2 expression was positive in 12 (85.7%) of the 14 patients with lymph node micrometastasis, and in 9 (56.3%) of the 16 patients without lymph node micrometastasis. However, the difference between these two groups was not statistically significant ($P = 0.118$) (Table 2).

DISCUSSION

The protein MMP-2 (type IV collagenase) belongs to the family of metalloproteinases. Its function is related to cellular matrix degradation including basement membrane type IV collagen. MMP-2 has been reported to play an important role in the metastatic process and tumor invasion of gastric carcinoma[16-19].

Monig *et al*[13] investigated correlations between MMP-2 immunoreactivity and currently used classification systems and possible relationships between lymph node metastasis and MMP-2 expression in a prospective study. MMP-2 expression correlated significantly with depth of tumor infiltration, lymph node metastasis, distant metastasis, and UICC stage. In contrast, no significant correlations were found between MMP-2 expression and other variables such as age, tumor differentiation, WHO, Lauren, Goseki, and Ming classifications. Sundblad *et al*[12] also reported that MMP-2 expression showed significant correlations with parietal depth of infiltration ($P = 0.03$) and with metastases in regional lymph nodes ($P = 0.05$). However, Ko *et al*[20] reported that MMP-2 expression was not correlated significantly with lymph node micrometastasis or depth of invasion ($P > 0.05$). They also reported that TIMP-2 expression (MMP-2 inhibitor) showed a strong relationship with lymph node micrometastasis and depth of tumor infiltration ($P < 0.05$).

In the present study, MMP-2 expression was observed in 70% (21 of 30 cases) of gastric carcinoma. MMP-2 expression increased in accordance with the depth of tumor invasion. MMP-2 expression was more frequent in T3/T4 tumors (8 of 9 cases, 88.9%) and T2 tumors (12 of16 cases, 75%), as compared with T1 tumors (1 of 5 cases, 20%). The difference was statistically significant ($P = 0.022$). In 20 gastric carcinomas with lymph node metastasis 17 (85%) showed positive MMP-2 expression, while in 10 gastric carcinomas without lymph node metastasis only 4 showed positive MMP-2 expression ($P = 0.030$). In addition, we also found that there was a significant correlation between MMP-2 expression and tumor differentiation. 88.2% (15 of 17 cases) poorly differentiated gastric carcinomas showed MMP-2 expression, while positive MMP-2 expres-

sion of moderately- and well-differentiated gastric carcinomas were only 50% (3 of 6 cases) and 42.9% (3 of 7 cases) respectively. The difference was also statistically significant ($P = 0.043$).

Our results demonstrate that MMP-2 expression has significant correlation with tumor invasion, tumor differentiation and lymph node metastasis. For gastric carcinoma, MMP-2 expression may be an important biological characteristic and a significant prognostic parameter.

Recent advances in immunohistochemistry and molecular biology techniques have made it possible to detect lymph node micrometastasis not evidenced by routine H-E staining. 28%-68.1% of patients with gastric carcinoma were reportedly identified micrometastases in regional lymph nodes[21-25]. However, the mechanism of lymph node micrometastasis is still not completely known now. Therefore, the main objective of this study was to examine MMP-2 expression in the tumor tissues and explore its relationship with lymph node micrometastasis. Totally, 77 (12.5%) lymph nodes of 14 (46.7%) patients with gastric carcinoma were detected lymph node micrometastases. In 14 gastric carcinoma patients with lymph node micrometastases 12 (85.7%) showed positive MMP-2 expression, while in 16 gastric carcinoma patients without lymph node micrometastases only 9 (56.3%) were examined MMP-2 expression. The results indicate that MMP-2 may participate in the development of lymph node micrometastasis of gastric carcinoma, however, the difference was not statistically significant ($P = 0.118$). This may be explained by the fact that the cases in our study were comparatively few. To draw a further conclusion, a large-scale investigation is needed.

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RAPID COMMUNICATION

Clinical value of CT three-dimensional imaging in diagnosing gastrointestinal tract diseases

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Abstract

AIM: To discuss the clinical value of CT three-dimensional (3-D) imaging in diagnosing gastrointestinal tract diseases.

METHODS: Three-D imaging findings of 52 patients were retrospectively analyzed. Three-D imaging methods included shaded surface display (SSD), volume rendering (VR), virtual endoscopy (VE) and multiplanar reformatting (MPR). The diagnosis results of CT 3-D were evaluated by comparison with those of endoscopy and/or surgical finding.

RESULTS: Fifty-two patients with gastrointestinal tract diseases were diagnosed by CT 3-D imaging, of whom 50 cases were correctly diagnosed and 2 were misdiagnosed. There were 33 cases of gastric diseases (27 with carcinoma, 5 with peptic ulcer and 1 with leiomyoma) and 19 large intestinal diseases (10 with colon carcinoma, 2 with carcinoma of the rectum, 5 with colon polypus and 2 with tuberculosis of the ileocecal junction). Twenty-two cases with prominent lesions (9 with subsequent hollow lesions), 20 with stenosis of cavity (8 with concomitant prominent lesions) and 10 with hollow lesions (5 with concomitant prominent lesions) were shown in 3-D images. The minimal lesion shown was 1.0 cm × 0.8 cm × 0.5 cm.

CONCLUSION: CT 3-D imaging, a non-invasive examination without pain, can display clearly and directly the lesions of gastrointestinal tract with accurate location and high diagnosis accuracy. It is an important complementary technique to endoscopy.

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INTRODUCTION

Barium studies and endoscopy are two conventional examinations of gastrointestinal tract diseases. In recent years, with the advancement of spiral CT (single-detector row spiral CT, SDCT) and multi-detector row spiral CT (MDCT) scanner, CT three-dimensional (3-D) imaging has gained wide application and high diagnosis accuracy in gastrointestinal tract diseases[1-4]. Here we report the results of CT 3-D imaging in diagnosing gastrointestinal tract diseases in our hospital, and evaluate its clinical application.

MATERIALS AND METHODS

General information

From Jan 2000 to Oct 2005, 52 patents with complete clinical data were examined by 3-D imaging (From Aug 2001 to Oct 2003, due to short supply of effervescent granules, no such examination was carried out), among whom 30 cases were males and 22 females. The average age of the patients was 57.3 years (range, 20-85 years). Thirty-three patients were examined in stomach and 19 in large intestine.

Examination instrument

SDCT scanner (Somatom plus/4, Siemens, Germany) and workstation with 3-D imaging software (SUN magicview 1000) were used in 28 cases (18 in stomach and 10 in large intestine). MDCT scanner (LightSpeed/16, GE, America) and workstation with 3-D imaging software (Advantage Workstation 4.2) were used in 24 cases (15 in stomach and 9 in large intestine).

Examination methods

All the patients were required to fast for 12 h. The patient examined in the stomach took one pack (3 g) of efferves-

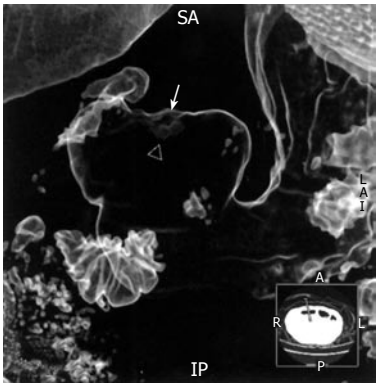


Figure 1 The ulcero carcinoma of stomach (on the anterosuperior wall of gastric antrum): the anterior-view of stomach on the VR image shows clearly the hollow lesion (↑) and the concomitant prominent lesion (△), with gas filling the stomach.

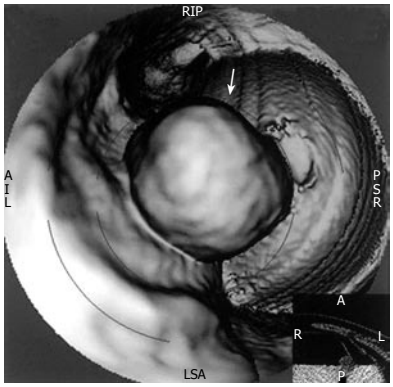


Figure 3 The leiomyoma of stomach (on the greater curvature of stomach): the axial-view of gastric cavity on VE image shows the prominent lesion (↑), with gas filling the stomach.

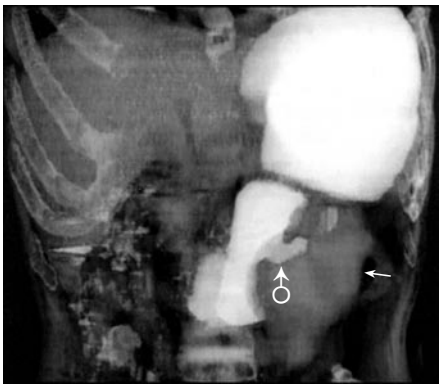


Figure 2 The ulcero carcinoma of stomach (on the greater curvature of stomach): the anterior-view of stomach on VR image shows the prominent lesion (↑) and the subsequent hollow lesion (♂), with 2% - 3% Omnipaque 300 filling the stomach.

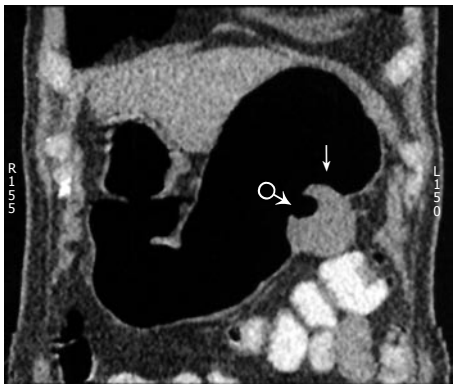


Figure 4 The same patient of **Figure 3**: the gastric coronal plane on MPR image shows the prominent lesion (↑) and the subsequent hollow lesion (♂).

cent granules (malu，Shanghai，China), lay in scanning bed and rolled to spread gas in stomach. A scout projection in supine position was made to confirm whether stomach was distended by gas. If distention was insufficient , another pack of effervescent granules (10 such cases in this study) or 500-1000 mL of 2%-3% Omnipaque 300 (Amersham, Shanghai，China) was added as an oral contrast agent to increase the contrasting effect and distending extent (8 such cases in this study). Another scout projection was scanned again. Holding breath for 10 s to 30 s, the patient was scanned with collimation of 5 mm (or 1.25 mm), pitch of 1.5 (or 1.375), increment of 2.4 mm (or 0.6 mm), scanning voltage of 120 kVp and a scanning current of 100-150 mA(the parameters in the brackets were used in scanning by MDCT). Another position of prone or lateral decubitus was often selected to better show some lesions (8 such cases in this study). The patients examined in the large intestines were asked to have a cleaning enema the night before and 30 min prior to the examination. A total of 600-1000 mL of air was infused through the anal canal as the contrasting agent. The following steps were the same as that used in stomach.

Image processing

Three-dimensional imaging methods included surface display (SSD), volume rendering (VR), virtual endoscopy (VE) and multiplanar reformatting (MPR). By selecting reconstruction thresholds of gastrointestinal walls from 15 Hu to 40 Hu and gastrointestinal cavities from -200 Hu to -1500 Hu, and necessary cutting technique, we obtained satisfactory 3-D images on the workstation. The exhibition of gastrointestinal tract lesions on the 3-D images was analyzed and measured, and the diagnosis results of CT 3-D imaging were evaluated by comparison with those of endoscopy and/or surgical finding.

RESULTS

Diseases diagnosed

Fifty-two patients with gastrointestinal tract diseases were histologically proven, of whom 33 cases had gastric diseases (27 with carcinoma, 5 with peptic ulcer and 1 with leiomyoma) and 19 had intestinal diseases (10 with colon carcinoma, 2 with carcinoma of rectum, 5 with colon polypus and 2 with tuberculosis of ileocecal junction). Fifty cases were correctly diagnosed (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6) and 2 misdiagnosed (1 with leiomyoma in stomach and 1 with tuberculosis in ascending colon).

Lesions shown

Prominent lesions (Figures 2-5) were shown in 3-D images in 22 cases (9 with subsequent hollow lesions, with maximal size of 5.4 cm × 6.0 cm × 2.1 cm and minimal of 1.5 cm × 1.2 cm × 0.8 cm), stenosis lesions (Figure 6) in 20 (8 with concomitant prominent lesions) and hollow lesions (Figure 1) in 10 (5 with concomitant prominent lesions, maximal of 2.0 cm × 1.8 cm × 1.1 cm, minimal of 1.0 cm × 0.8 cm × 0.5 cm). The locations, sizes and shapes of lesions in the 3-D images were close to that seen under endoscopy and/or in surgery.

Characteristics of imaging methods

Three-D images with VR, VE, SSD and MPR could

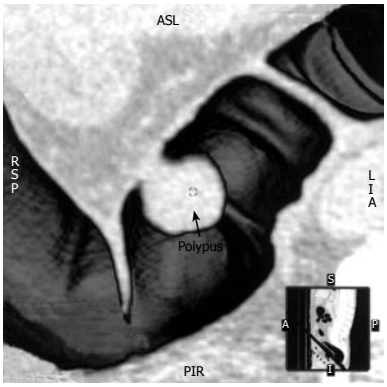


Figure 5 The polypus of colon: the anterior-view of colon on SSD image, by using the cutting technique, shows the prominent lesion (↑), with air filling the large intestine.



Figure 6 The carcinoma of descending colon: the anterior-view of large intestine on VR image shows the stenosis of intestinal cavity (↑), with air filling the large intestine.

show gastrointestinal tract lesions (Table 1), each with its own characteristics. SSD-imaging should be made with necessary cutting technique (Figure 5). VE-imaging could only show the extraluminal lesions. MPR-imaging had no perception of 3-D, but it could show clearly wall invasion (Figure 4). VR-imaging could show both extraluminal and intraluminal lesions (Figures 2, 6).

Table 1 Gastrointestinal tract lesions by3-D imaging with SSD, VR, VE and MPR

Methods	Prominence ($n=22$)			Hollow ($n=10$)			Stenosis ($n=20$)		
	G	M	B	G	M	B	G	M	B
SSD, cutting	18	4	0	7	2	1	19	1	0
V R	20	2	0	9	1	0	19	1	0
V E	20	2	0	6	3	1	17	3	0
MPR	18	4	0	4	4	2	18	2	0

G-good, indicates the sign was clear; M-medium, identifiable; B-bad, unclear.

DISCUSSION

Characteristics in showing lesions

Gastrointestinal tract lesions could be clearly shown by 3-D imaging, and the exact location and accurate size of a lesion could be obtained when it was more than 5 mm. In our study, the minimal lesion shown by 3-D image was 1.0 cm × 0.8 cm × 0.5 cm. This result is accordant with that reported by Zhang and Gluecker[3,5]. The main advantages of 3-D imaging over two-dimensional (2-D) multiplanar reformatting are added perspective and enhanced depth perception. The relationship between the lesion and adjacent structures can be better appreciated. It is often helpful to use the combinations of different 3-D imaging methods in different lesions, and to start with 2-D multiplanar reformatting and then to proceed to 3-D imaging[6]. Therefore in order to increase the diagnostic accuracy, we suggest several 3-D images be observed as well as the original axial images.

Influence of examination techniques on 3-D image

Theoretically, the quality of 3-D images is related to slice thickening, pitch and tube current[7]. Our study found that a satisfactory 3-D image of increased spatial resolution and decreased moving artificial shadows was obtained with thinner collimations and appropriate tube current and pitch. Interference of the contents in the gastrointestinal tract was avoided and adequate distention was obtained by using gas from effervescent granules of air as the contrasting agent of gastrointestinal tract, or by setting the patient in the prone, lateral decubitus and left posterior oblique position[8]. If gastrointestinal tract is not well distended or gastrointestinal wall has collapsed, some lesions may be overlooked and some similar disease may appear[9]. Traditionally, low or high attenuation contrasting agent can both be taken to enhance and distend the gastrointestinal tract. As to whether to take low or high attenuation contrasting agent, we think low-attenuation, e.g. gas, should be the first choice, as the agents are safer, well tolerated, and always result in good gastrointestinal tract distention. 2%-3% Omnipaque 300 may be optimal when stomach cannot be well distended. Horton *et al*[1,10] reported low-attenuation agents, e.g. oil-based oral contrast agents, whole milk and water, resulted in satisfactory evaluation of detection of subtle diseases of the gastric wall, and did not interfere with the manipulation of 3-D data sets. It is necessary to conduct further study on the clinical application of oral contrast agents.

Comparisons among 3-D imaging methods

All the 3-D imaging methods could show the lesions of gastrointestinal tracts, each with its own characteristics: MPR image could show the lesion in any section, e.g. sagittal or oblique. Though it could not get 3-D perception, it could remedy the defects of transverse-images, with the advantages of showing the gastrointestinal lesions of wall invasion. Images of VE and SSD with cutting techniques can show the size and shape of the lesions[6], especially intraluminal prominent lesions, and show the structure of gastrointestinal tracts, e.g. semilunar fold and ileocecal valve, from any directions without being influenced by a narrow cavity. They can better show the base and pedicel of lesions, but cannot discern surface coloration of lesion and extraluminal tumor invading [11]. VR images can be made translucent, similar to those of double contrast barium studies, and can demonstrate the overall contour changes of gastrointestinal tracts, but with poor mucosal information[12].

Clinical value of 3-D imaging

Accurate staging of gastrointestinal cancer is essential because surgical resection is the treatment only for local-

ized disease. Endoscopy and conventional barium study can only detect intraluminal lesions, but cannot evaluate extraluminal lesions. Traditional CT images can show anatomic structure and lesions of the gastrointestinal tract, but lack continuity and three dimensions. CT 3-D imaging has many advantages in the staging of primary tumor, assessing for local spread and detecting nodal involvement and distant metastases. It can provide valuable additional information and improve the detection and staging of both early and advanced gastric tumors and the detection of colorectal cancer and polypus[1,13,14]. CT 3-D imaging, with the same volume data obtained by spiral CT, especially by MDCT, can be repeatedly made with different reconstruction methods and avoid leakage of the section and moving artificial shadows. It has a high accuracy, sensitivity and specificity in showing prominent lesions and stenosis lesions, and in diagnosing gastric carcinoma and colon carcinoma[15]. 3-D imaging is a non-invasive examination without pain, and can easily be accepted by patients[14]. It may be used as an important complementary technique to endoscopy, particularly when the cavity is too narrow for endoscopy to go through. However, 3-D imaging examination has its limitations, e.g. it cannot show the color of lesions or make bioscopy[2].

Summary

CT 3-D imaging can clearly and directly show lesions of gastrointestinal tract. Diagnostic accuracy can be improved by observing different 3-D images together with original 2-D images. Three-D imaging is a non-invasive examination without pain, with characteristics of accurate localization of lesions and comprehensive observation of shapes. It is an important complementary technique to endoscopy.

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CASE REPORT

Successful aspiration and ethanol sclerosis of a large, symptomatic, simple liver cyst: Case presentation and review of the literature

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Abstract

Simple liver cysts are congenital with a prevalence of 2.5%-4.25%. Imaging, whether by US, CT or MRI, is accurate in distinguishing simple cysts from other etiologies, including parasitic, neoplastic, duct-related, and traumatic cysts. Symptomatic simple liver cysts are rare, and the true frequency of symptoms is not known. Symptomatic simple liver cysts are predominantly large (> 4 cm), right-sided, and more common in women and older patients. The vast majority of simple hepatic cysts require no treatment or follow-up, though large cysts (> 4 cm) may be followed initially with serial imaging to ensure stability. Attribution of symptoms to a large simple cyst should be undertaken with caution, after alternative diagnoses have been excluded. Aspiration may be performed to test whether symptoms are due to the cyst; however, cyst recurrence should be expected. Limited experience with both laparoscopic deroofing and aspiration, followed by instillation of a sclerosing agent has demonstrated promising results for the treatment of symptomatic cysts. Here, we describe a patient with a large, symptomatic, simple liver cyst who experienced complete resolution of symptoms following cyst drainage and alcohol ablation, and we present a comprehensive review of the literature.

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Key words: Simple hepatic cyst; Alcohol sclerosis; Laparoscopic deroofing

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INTRODUCTION

Liver cysts are classified as true or false, depending on the presence of an epithelial lining[1]. True cysts include congenital cysts (simple cysts and polycystic liver disease), parasitic (hydatid) cysts caused by *Echinococcus granulosis* and *multilocularis* tapeworms, neoplastic cysts (including cystadenoma, cystadenocarcinoma, cystic sarcoma, squamous cell carcinoma, and metastatic ovarian, pancreatic, colon, renal and neuroendocrine cancers), and biliary duct-related cysts (Caroli's disease, bile duct duplication, and peribiliary cysts)[1]. False cysts may be caused by spontaneous intrahepatic hemorrhage, post-traumatic hematoma, or intrahepatic biloma[1].

The pathogenesis of liver cysts is not clear. Simple liver cysts are congenital[2]. They are lined by cuboidal epithelium and originate from the abnormal development of intrahepatic ducts *in utero*. They are generally stable in size over time, but may slowly enlarge and occasionally become symptomatic due to mass effect, rupture, hemorrhage, or infection[3]. However, an enlarging cyst should prompt consideration of diagnosis other than simple cysts. Although simple cysts are generally solitary, more than one cyst may be present ("several solitary"), even in the absence of polycystic liver disease, as is the case with the patient described below.

We report herein a case of a patient with a large, symptomatic, simple hepatic cyst with resolution of symptoms immediately after therapy. In addition, we present a comprehensive literature review of diagnosis and treatment options of symptomatic hepatic cysts.

CASE REPORT

A 59-year-old African-American woman was referred to the Division of Gastroenterology at the University of Pennsylvania for evaluation of abdominal pain for 2 years. The patient described frequent, intermittent epigastric pain and bloating not associated with meals or bowel movements. Typically, pain would last for one-half hour before spontaneously resolving. She had twice presented to the emergency room with abdominal pain



Figure 1 Enhanced abdominal CT scan showing large, simple hepatic cyst.

and was discharged without definitive diagnosis. She also complained of early satiety and occasional nausea without weight loss.

The patient was taking daily fiber supplements and had regular, daily bowel movements. Reflux symptoms were well controlled with esomeprazole. Hypertension was treated with metoprolol and amlodipine. Other chronic medications were progesterone and estrogen. She denied non-steroidal anti-inflammatory drug usage. Physical examination was unremarkable.

Laboratory evaluation showed normal liver-associated enzymes, metabolic panel, complete blood count, and urinalysis. Screening colonoscopy, performed two years prior to presentation, revealed internal hemorrhoids and melanosis. Upon presentation, she underwent double contrast upper gastrointestinal examination, which suggested antral gastritis and a hiatal hernia. On subsequent upper gastrointestinal endoscopy, the mucosa appeared normal, and a medium-sized hiatal hernia was present. Treatment with a proton pump inhibitor, fiber, and hyoscyamine failed to improve symptoms by six months.

The patient underwent abdominal CT scan with intravenous and oral contrast, which showed a large (7.7 cm) hepatic cyst in the lateral segment of the left lobe, as well as several other smaller cysts (Figure 1). Gallstones without gallbladder wall thickening or pericholecystic fluid were also visualized. On ultrasonography, tenderness was elicited specifically over the site of the cyst, which measured 10.3 cm in its longest dimension.

After several months of expectant management, the patient was referred to interventional radiology for drainage of the large hepatic cyst. Under ultrasonographic guidance, an 8-gauge French catheter was placed into the cyst, serous non-bilious fluid was aspirated, and the catheter was placed to gravity drainage. One week later, after drainage had ceased, the patient underwent ethanol sclerosis. At 4 mo follow-up, the patient was completely symptom-free. Follow-up ultrasound showed complete cyst resolution.

DISCUSSION

Previous studies, based on autopsy and surgical series, estimated a very low prevalence of simple non-parasitic hepatic cysts (0.14%-0.17%)[4]. More recently, among patients referred for abdominal ultrasonography, the prevalence of simple hepatic cysts has been reported 2.5%-4.65%[5,6]. Liver cysts have been recognized increasingly as the routine use of imaging studies becomes more widespread. Hepatic cysts may be more common in women[5] and in patients older than 40 years[6]. Symptoms, though quite rare, may be related to the space-occupying effect of large cysts[7] and may be more common in right-sided cysts[8]. Symptoms may include abdominal discomfort, chronic right upper quadrant or epigastric abdominal pain, early satiety, dyspnea, increasing abdominal girth, nausea, and vomiting[7,9]. Sanfelippo *et al*[4] reported that among 15 symptomatic patients with solitary non-parastic liver cysts, abdominal mass was present in 54%, hepatomegaly in 40%, abdominal pain in 33% and jaundice in 9% patients.

Table 1 Rare complications of simple liver cysts

Obstructive jaundice
Infection
Intracystic haemorrhage
Spontaneous rupture
Inferior vena cava obstruction
Neoplastic transformation
Primary squamous cell carcinoma
Cystadenocarcinoma
Adenosquamous carcinoma
Adenocarcinoma
Hepatocellular carinoma
Cholangiocarcinoma

Although the natural history of simple hepatic cysts is not well known, complications appear to be quite rare (Table 1). Obstructive jaundice caused by solitary non-parasitic liver cyst is rare[11-15], and such cysts are usually large and located centrally in the liver, causing compression of the hepatic hilum[13]. However, sometimes even small hepatic cysts (3 cm in diameter) may cause common bile duct stenosis and intrahepatic biliary dilatation[15].

Liver cysts may also cause obstruction of the inferior vena cava, which may lead to massive edema of the legs[16,17] and scrotum[16]. Infections of simple hepatic cysts with *Klebsiella pneumoniae*[18] and *Escherichia coli*[19], presenting with acute onset of right upper quadrant abdominal pain, diarrhea, and fever have also been reported. Other documented complications include intracystic haemorrhage[19-23] and spontaneous rupture[23]. Neoplasms arising from solitary non-parasitic liver cysts, including primary squamous cell carcinoma[24-26], cystadenocarcinoma, adenosquamous carcinoma, adenocarcinoma, hepatocellular carinoma, and cholangio carcinoma[27-29], have been reported, but appear to be very rare. A possible association with Peutz-Jeghers syndrome has also been suggested[30].

Imaging modalities (ultrasound, CT, and MRI) are highly accurate for diagnosing simple cysts (Table 2). Large differences in echogenicity between hepatic parenchyma and cyst fluid allow for easy recognition of simple cysts by ultrasound[31]. The ultrasonographic appearance of simple cyst is characterized by well-defined, echo-free lesions with

Table 2 Radiologic features of simple hepatic cysts

Features supporting diagnosis of a simple cyst	Features not supporting diagnosis of a simple cyst
Anechoic lesion	Echoic lesion
Thin wall	Thick wall
Absence of septations	Presence of septations
No peripheral enhancement on CT/MRI	Peripheral enhancement on CT/MRI
Homogeneous appearance	Heterogeneity within the cyst
	Hydatid sand
	Presence of daughter cysts
	Heavy wall calcifications

Table 3 Alternative explanations for symptoms in patients with simple hepatic cysts

Diagnosis
Biliary colic
Gastroesophageal reflux
Peptic ulcer
Non-ulcer dyspepsia
Irritable bowel syndrome
Chronic pancreatitis
Abdominal wall pain syndrome

good through transmission and an imperceptible wall[3,32]. The presence of acoustic enhancement results from relative lack of absorption and reflection of sound by cyst fluid, as compared with hepatic parenchyma[32]. On CT scan, simple cysts appear as well-demarcated, water-density sacs, which do not demonstrate peripheral enhancement after intravenous contrast [3]. The presence of septations suggests that a cyst is not simple. Occasionally, large simple cysts may have "septations" due to hemorrhage. MRI shows simple cysts as hypointense lesions on T1-weighted and hyperintense on T2-weighted images[32,33]. Simple cysts differ from cavernous hemangiomas in that they are more hypointense on T1-weighted and of equal hyperintensity on T2-weighted images[33]. Radiologic characteristics which would argue against a cyst being simple include a thick wall, peripheral enhancement on CT or MRI, heterogeneity within the cyst, and an increase in size over time. Hepatic cysts should be differentiated from hepatic abscesses, hematomas, necrotic metastases, an intrahepatic gallbladder, biliary cystadenoma, and echinococcal (hydatid) cysts[32].

Radiologic imaging can accurately identify hydatid cysts, with an accuracy of 96% in one series[34]. Presence of hydatid sand, internal sepatations, daughter cysts, and heavy wall calcifications argue for *Echinococcus granulosis* infection, instead of a simple cyst. Epidemiologic features and serology in combination with radiologic imaging generally lead to the correct diagnosis non-invasively.

Distinction between cystic and solid hepatic lesions can be made accurately by ultrasonography, though CT or MR imaging may be more sensitive for the detection of focal hepatic masses[35]. The presence of any peripheral enhancement or thick-walled component suggests the possibility of hepatic abscess or neoplasm[32]. Small hepatic lesions with diameter < 1 cm are difficult to classify[36]. Such lesions should be differentiated from benign cysts, hepatic metastases with central necrosis, and microabscesses[36]. In order to differentiate small cysts from small metastases, follow-up imaging may be considered in selected cases[36].

The vast majority of simple hepatic cysts require no treatment. Large cysts (diameter of 4 cm or more) can be followed for stability with repeated imaging[37]. If the cyst remains unchanged for 2 years, further monitoring may be discontinued[37]. Symptomatic or enlarging cysts require consideration of alternative diagnoses, including cystadenoma, cystadenocarcinoma, and hepatic metastases. It should be stressed that attribution of symptoms to simple cysts should be undertaken with caution after excluding alternative diagnoses. Epigastric or right upper quadrant abdominal pain provoked by eating may indicate biliary colic, if gallstones are present. A successful trial of acid suppression therapy points to gastroesophageal reflux disease. Selected patients may undergo upper gastrointestinal endoscopy to diagnose erosive esophagitis or peptic ulcer disease. Esophageal pH monitoring can confirm the diagnosis of gastroesophageal reflux. If symptoms fluctuate in concert with changes in stool frequency or form, a diagnosis of irritable bowel syndrome should be suspected. Finally, a diagnosis of non-ulcer dyspepsia may be entertained in patients with unremarkable upper gastrointestinal endoscopy who have continued prominent upper abdominal pain, possibly in association with nausea and vomiting (Table 3). If the preceding diagnoses can be confidently excluded, then treatment of a large, symptomatic hepatic cyst may be undertaken. Treatment options include needle aspiration with or without injection of sclerosing solution, internal drainage with cystojejunostomy, wide deroofing, and different degrees of liver resection[37].

Percutaneous US- or CT-guided needle aspiration of hepatic cysts is associated with high recurrence rates (78%-100%)[38,39]. Several small case series have demonstrated efficacy for the performance of US- or CT-guided needle aspiration of hepatic cysts combined with alcohol injection[40-44]. Because US-guided aspiration with ethanol sclerosis is generally safe, effective, and relatively non-invasive, it may be a first-line treatment for selected symptomatic congenital hepatic cysts, especially in patients with high surgical risk or polycystic liver disease[15]. The 95%, 96% and 99% alcohol solutions are equally safe and effective[15,42,45]. Enough alcohol should be instilled to replace 25% of the aspirated cyst fluid volume[46]. For larger cysts (> 400 mL), multiple alcohol injections in the same sitting have been proposed[46]. Alcohol fixes the cells lining the cyst cavity, disabling their ability to secrete fluid and promote cyst enlargement[46]. Recurrence may occur if alcohol does not come in contact with all cells lining the cyst cavity[46] and may be more common in uncooperative patients[47]. Combinations of percutaneous aspiration with other sclerosing substances, such as iophendylate (pantopaque)[48], tetracycline chloride[49-52], doxycycline[52], minocycline chloride[53-56], and hypertonic saline solution[8] have also achieved good outcomes.

Several recent series have demonstrated good results for laparoscopic deroofing procedures. Widest possible excision of the cystic wall and concomitant argon beam

Table 4 Comparison of treatment options for symptomatic simple liver cysts

Treatment options	Advantages	Disadvantages
Observation alone	- Because most cysts are asymptomatic, intervention is unlikely to be helpful and may be harmful	- Only effective cyst treatment can prove whether symptoms are related to the cyst
US-guided aspiration	- Simple procedure - May be used as a diagnostic test to assess whether symptoms are related to the cyst	- High recurrence rate
US-guided aspiration with sclerotherapy	- Relatively non-invasive - Complications are rare - Effective - Possible in poor surgical candidates	- Less effective for uncooperative patients - Can not be performed if cyst communicates with biliary tree
Laparoscopic unroofing	- Technically feasible and effective in > 80% cases - Improved results with extensive fenestration and argon beam coagulation or electrocoagulation - Low recurrence rate (0%-20%) - Visualization of cyst interior (exclude other diagnoses)	- More invasive - Morbidity in up to 25% - Less effective for cysts which are superior, posterior, or deep within hepatic parenchyma - Less effective if prior surgery has been attempted
Laparotomy (resection, fenestration, or excision)	- Effective - Allows treatment of laparoscopically inaccessible cysts - Useful for cysts with complications - May perform cystojejunostomy at time of laparotomy for cysts with biliary communication	- Most invasive - Larger scars - Longer hospital stays compare to laparoscopy - Significant post-surgical morbidity

coagulation or electrocoagulation may improve the durability of results[57-61]. Recurrence has ranged from 0% to 20% with morbidity in 0% to 25%[57-60,62]. In one series, laparoscopic fenestration of simple hepatic cysts was found to be technically feasible in 90% with symptomatic relief in 95% during 38.5 mo follow-up[61]. Careful selection of patients who have not previously undergone surgical treatment and have large, symptomatic, superficial, and anterior cysts may improve outcomes[63]. US- or CT-guided cyst aspiration can be performed prior to laparoscopic fenestration in selected cases to assess whether symptoms are truly referable to the cyst[63]. Reported complications associated with laparoscopic deroofing include wound infection, bile leak, chest infection, subphrenic hematoma, and prolonged post-procedure drainage[37]. In selected cases, an open surgical procedure (fenestration, excision, or resection) may be preferred, despite longer recovery times and larger surgical scar, because of cyst location, surgeon expertise, or the presence of complicating factors[8,39,61,63,64].

Laparoscopic deroofing and cyst aspiration, followed by sclerosis, are both reasonable approaches for the majority of symptomatic simple liver cysts (Table 4)[64]. Whereas sclerosis may be less invasive and associated with lower rates of complications, laparoscopy is effective and provides the opportunity to directly examine the cyst interior to rule out etiologies other than a simple hepatic cyst[64,65].

It is important to rule out biliary communication before sclerosing a cyst, as irreversible sclerosing cholangitis has been reported as a complication[7,16,46,52,66]. The presence of cystobiliary communication may be established by endoscopic retrograde cholangiography or aspiration of bile-stained cystic fluid and is often treated by open cystojejunostomy[39], although the cystobiliary communication may be closed laparoscopically[67].

In summary, we present the case of a large, symptomatic simple hepatic cyst. We established that the cyst was the cause of symptoms only after systematically excluding alternative diagnoses through testing and empiric therapy. The usual standard of care for patients with large, simple hepatic cysts is observation, but our case demonstrates that large cysts can occasionally be responsible for symptoms. In selected cases, symptoms may respond to cyst treatment. Our treatment approach was ultrasound-guided aspiration and ethanol sclerotherapy. We believe that aspiration alone is associated with unacceptably high rates of recurrence. In our patient, excellent symptomatic and radiologic responses were achieved. In the case of cyst recurrence laparoscopic deroofing may be considered, though complications may occur in up to 25% of cases.

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CASE REPORT

Autoimmune pancreatitis metachronously associated with retroperitoneal fibrosis with IgG4-positive plasma cell infiltration

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Abstract

Retroperitoneal fibrosis is an uncommon disorder characterized by the formation of a dense plaque of fibrous tissue in the retroperitoneum, and its etiology remains unknown. Autoimmune pancreatitis is a rare type of chronic pancreatitis characterized by fibrosis with abundant infiltration of IgG4-positive plasma cells and lymphocytes and obliterative phlebitis in the pancreas. We present a case of autoimmune pancreatitis that developed 10 mo after the occurrence of retroperitoneal fibrosis. Histological findings of the resected retroperitoneal mass were marked periureteral fibrosis with abundant infiltration of IgG4-positive plasma cells and lymphocytes and obliterative phlebitis. These findings suggest a common pathophysiological mechanism for retroperitoneal fibrosis and autoimmune pancreatitis in this case. Some cases of retroperitoneal fibrosis might be a retroperitoneal lesion of IgG4-related sclerosing disease.

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Key words: Autoimmune pancreatitis; Retroperitoneal fibrosis; IgG4

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INTRODUCTION

Retroperitoneal fibrosis is an uncommon disorder characterized by the formation of a dense plaque of fibrous tissue in the retroperitoneum. Although retroperitoneal fibrosis leads to ureteral encasement and consequently hydronephrosis in most cases, it may also involve other structures in the retroperitoneum as well as in multiple unattached sites. Retroperitoneal fibrosis may be part of a systemic fibrosing disease[1,2]. Although the exact etiology of retroperitoneal fibrosis remains unknown, some cases respond well to steroid therapy[2,3].

Autoimmune pancreatitis is a recently noted clinical entity and a rare group of pancreatitis in which autoimmunity is suspected as a pathogenic mechanism. It is characterized by enlargement of the pancreas, irregular narrowing of the main pancreatic duct, elevated serum IgG4 concentrations, favorable response to steroid therapy and lymphoplasmacytic infiltration with fibrosis in the pancreas[4,5]. Recent studies have revealed occasional coexistence of autoimmune pancreatitis with other systemic exocrinopathy, such as sclerosing cholangitis[6] or sclerosing sialadenitis[7]. We have previously reported 2 other cases of autoimmune pancreatitis associated with retroperitoneal fibrosis[8]. Here, we report a case of autoimmune pancreatitis that occurred 10 mo after retroperitoneal fibrosis. We also focus on the common histopathological features with abundant IgG4-positive plasma cell infiltration in both the pancreas and retroperitoneal mass.

CASE REPORT

A 75-year-old male was admitted to another hospital with a complaint of back pain. Abdominal computed tomography (CT) showed left hydronephrosis (Figure 1A) caused by a retroperitoneal mass with encasement of the ureter (Figure 1B), which was highly suggestive of a ureteral tumor. Resection of the left lower ureter and ureterostomy were performed. Histological examination of the retroperitoneal mass revealed marked periureteral fibrosis with dense perivascular infiltration of lymphocytes and plasma cells (Figure 2A) and obliterative phlebitis (Figure 2B). These findings were compatible with retroperitoneal fibrosis. Ten months later, jaundice with abdominal pain appeared, and he was referred to our hospital for further examination.

Laboratory studies on admission were as follows: total serum bilirubin 10.4 mg/dL (normal range: 0.2-1.1 mg/dL), alkaline phosphatase 1418 IU/L (115-359 IU/L), γ-glutamyl trasnpeptidase 558 IU/L (9-70 IU/L), glutamic-

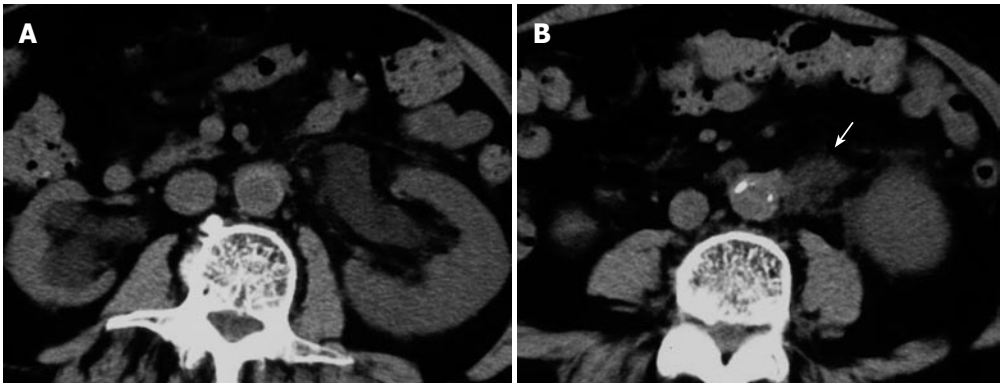


Figure 1 CT performed performed in another hospital showing left hydronephrosis (**A**) caused by a retroperitoneal mass (arrow) (**B**).

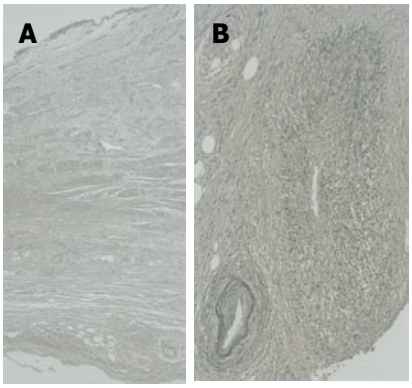


Figure 2 Histological findings of the retroperitoneal mass showing marked periureteral fibrosis with dense perivascular infiltration of lymphocytes and plasma cells (**A**, H&E) and obliterative phlebitis (**B**, Elastica Van Gienson stain).

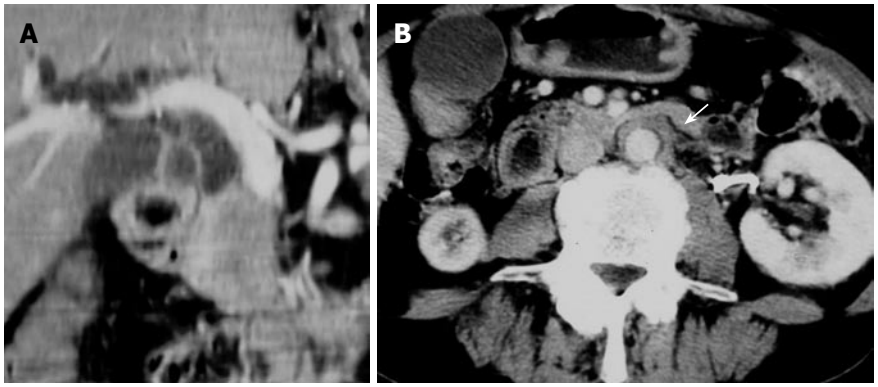


Figure 3 CT performed in our hospital showing the lower common bile duct obstructed with focally swollen pancreatic head (**A**) and soft tissue surrounding the aorta (arrow) (**B**).

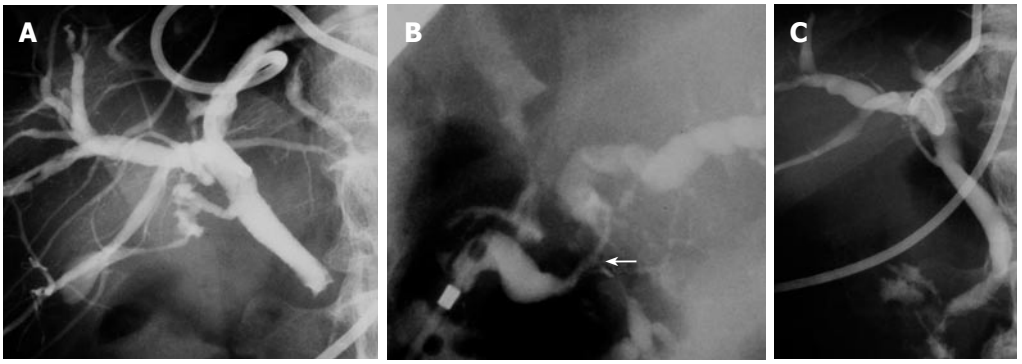


Figure 4 Percutaneous transhepatic cholangiography showing complete obstruction of the lower common bile duct (**A**) and resolution of the bile duct after steroid therapy (**C**). Endoscopic retrograde cholangiopancreatography demonstrating segmental narrowing of the main pancreatic duct in the pancreatic head (arrow) (**B**).

oxaloacetic transaminase 277 IU/L (12-32 IU/L), amylase 79 IU/L (45-155 IU/L), and elastase-1 1313 ng/mL (72-432 ng/mL). Tumor marker levels were within normal range. Although serum IgG level was 1380 mg/dL (870-1 700 mg/dL), serum IgG4 levels was elevated to 240 mg/dL (< 70 mg/dL). Antinuclear antibody and rheumatoid factor were negative. CT showed the lower common bile duct obstructed with focally swollen pancreatic head (Figure 3A) and soft tissue surrounding the aorta (Figure 3B). Magnetic resonance cholangiopancreatography (MRCP) showed the stricture of the lower common bile duct with dilatation of the upper common bile duct, but the pancreatic duct was not visualized. Percutaneous transhepatic biliary drainage was performed, and the common bile duct was completely obstructed in the lower portion (Figure 4A). Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated segmental narrowing of the main pancreatic duct in the pancreatic head (Figure 4B). Biopsy specimen taken from the major duodenal papilla during duodenoscopy revealed severe lymphoplasmacytic infiltration. Immunohistochemical studies using anti-IgG4 antibody (Binding Site, Birmingham, UK) with avidin-biotin-peroxidase complex (ABC) revealed abundant infiltration of IgG4-positive plasma cells in tissue specimens of both resected retroperitoneal mass (Figure 5A) and biopsied major duodenal papilla (Figure 5B). We diagnosed autoimmune pancreatitis and treated him with 30 mg predonisolone daily for 2 wk. The dose was

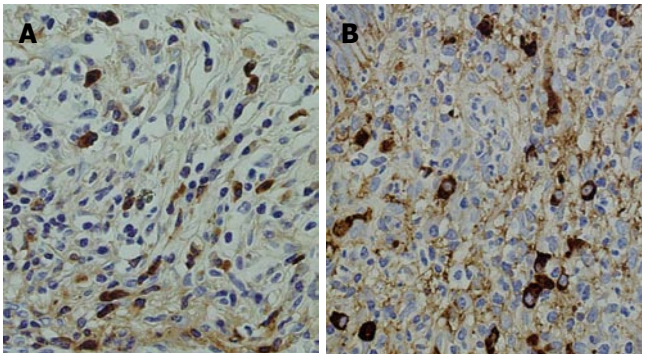


Figure 5 IgG4 immunostaining showing abundant infiltration of IgG4-positive plasma cells in tissue specimens of resected retroperitoneal mass (**A**) and biopsied major duodenal papilla (**B**).

tapered by 2.5-5 mg every 1-2 wk until a daily maintenance dose of 5 mg. After steroid therapy, findings on cholangiopancreatography and CT improved (Figure 4C), and the biliary drainage tube could be withdrawn. Serum IgG4 lowered to 83 mg/dL.

DISCUSSION

We have previously reported 2 other cases of autoimmune pancreatitis associated with retroperitoneal fibrosis[8]. A total of 9 cases including the present case have been reported[8-12]. All were middle-aged or elderly males. Autoimmune pancreatitis was synchronous to retroperitoneal fibrosis in 4 cases, and metachronous to retroperitoneal fibrosis in 3 cases. Retroperitoneal fibrosis was metachronous to autoimmune pancreatitis in 2 cases. Steroid therapy was effective for both pancreatic and retroperitoneal lesions.

Histological findings characteristic to autoimmune pancreatitis are dense lymphoplasmacytic infiltration with fibrosis and obliterative phlebitis in the pancreas[3,4]. We have reported that this lymphoplasmacytic infiltration with fibrosis is observed in the peripancreatic retroperitoneal tissue, biliary tract, and salivary glands, as well as in the pancreas of patients with autoimmune pancreatitis. Furthermore, an abundant infiltration of IgG4-positive plasma cells was observed in the various organs of patients with autoimmune pancreatitis, including the peripancreatic retroperitoneal tissue, biliary tract, salivary glands, gastric mucosa, lymph nodes, and pancreas, but an abundant infiltration of IgG4-positive plasma cells was not detected in the organs of patients with other diseases[13,14]. Major duodenal papilla also showed fibrosis with abundant infiltration of IgG4-positive plasma cells[15]. We proposed the existence of a novel clinicopathological entity, an IgG4-related systemic disease characterized by a preponderance for elderly male, and extensive IgG4-positive plasma cell and T lymphocyte infiltration of organs[14]. We also suggest that autoimmune pancreatitis is not simply a pancreatitis but that, in fact, it is a pancreatic lesion reflecting an IgG4-related systemic disease, and that in some cases only organs other than the pancreas might be clinically involved[14].

In the present case, the retroperitoneal mass, that occurred 10 mo prior to autoimmune pancreatitis, histologically showed marked periureteral fibrosis with abundant infiltration of IgG4-positive plasma cells and lymphocytes and obliterative phlebitis, which were similar findings to those in the pancreas and peripancreatic retroperitoneal tissues of patients with autoimmune pancreatitis. These findings suggest a common pathophysiological mechanism for retroperitoneal fibrosis and autoimmune pancreatitis in this case. Although the exact etiology of retroperitoneal fibrosis remains unknown, some cases might be a retroperitoneal lesion of IgG4-related sclerosing disease.

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CASE REPORT

Gastric signet-ring adenocarcinoma presenting with breast metastasis

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Abstract

Breast metastases from gastric cancer are extremely rare. A case report of a 37-year-old female with breast inflammatory invasion and ascites is described. Breast biopsy revealed carcinomatous invasion of the lymphatics from adenocarcinoma cells with signet-ring features. Estrogen (ER) and progesterone receptors (PR) and c-erb-B2 were negative. Upper gastrointestinal endoscopy revealed a prepyloric ulcerative mass. Histopathologic examination of the lesion showed infiltration from a high-grade adenocarcinoma, identical with that of the breast. Immunostaining was positive for cytokeratins CK-7 and CK-20 and CEA and negative for ER and PR. Ascitic fluid cytology was positive for adenocarcinoma cells. Mammography was not diagnostic. Abdominal CT scanning revealed large ovarian masses suggestive of metastases (Krukenberg's tumor). A cisplatin-based regimen was given but no objective response was observed. The patient died six months after initial diagnosis. A review of the literature is performed.

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Key words: Gastric adenocarcinoma; Signet-ring; Breast metastasis; Ovarian metastasis

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INTRODUCTION

Breast metastases from extra-mammary neoplasms are extremely rare. The incidence ranges between 1%-2% in clinical and 1.7%-6.6% in autopsy series[1-2]. Less than 500 cases of secondary breast tumors have been reported so far in the English speaking literature[3]. The most common primary malignancies metastasizing to the breast are malignant melanoma, lung cancer, carcinoid tumors, ovarian cancer, renal cancer, gastrointestinal carcinomas and a variety of other primaries[2-4]. Herein, a case of a woman with gastric signet-ring adenocarcinoma presenting with breast metastasis is described.

CASE REPORT

A 37-year-old female presented to our department in September 2003 with abdominal distention and painful and edematous left breast. Her medical history was unremarkable except for three attempts of *in vitro* fertilization during the last two years. Physical examination revealed ascites and marked left breast inflammation. Neither discrete tumor nodules in the breast nor any enlarged axillary nor supraclavicular lymph nodes were palpable. Mammography showed diffuse increased density and skin thickening, without evidence of mass or microcalcifications. Abdominal ultrasonography established the presence of bilateral cystic adnexal masses with thickened walls and ascitic fluid. Abdominal CT confirmed the presence of large ovarian masses suggestive of metastases (Krukenberg's tumor); however, there was no evidence of lung, liver or bone metastases. Serum tumor markers CEA, CA 15-3 and CA 19-9 were within normal limits and CA 125 was slightly elevated (63.6 U/L). Ascitic fluid cytology was found positive for adenocarcinoma. Core needle biopsy of the left breast revealed diffuse infiltration of the lymphatics in form of tumor emboli from neoplastic cells with signet-ring features (Figure 1). Immunohistochemical studies for estrogen receptor (ER) and progesterone receptor (PR) as well as for c-erb-B2 were negative. Upper gastrointestinal endoscopy demonstrated a large prepyloric ulcerative mass. Biopsy of the lesion revealed infiltration from a diffuse-type high-grade gastric adenocarcinoma with signet-ring features (Figure 2 and Figure 3). Malignant cells were positive for cytokeratins 7 and 20 (CK7, CK20) and CEA, and negative for ER and PR. Subsequent study of the breast biopsy revealed similar findings (positive staining for CK7, CK20 and CEA). The patient was treated with cisplatin-based chemotherapy; but she showed no objective response and died six months after the initial diagnosis.

DISCUSSION

Metastatic breast involvement from extra-mammary

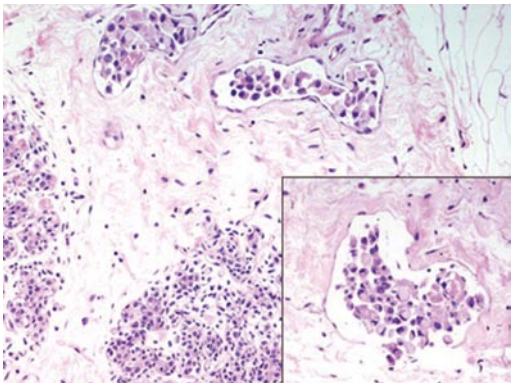


Figure 1 Breast biopsy showing breast lymphatic invasion from neoplastic cells with signet-ring features (Hematoxylin & eosin, 200 ×, 400 ×).

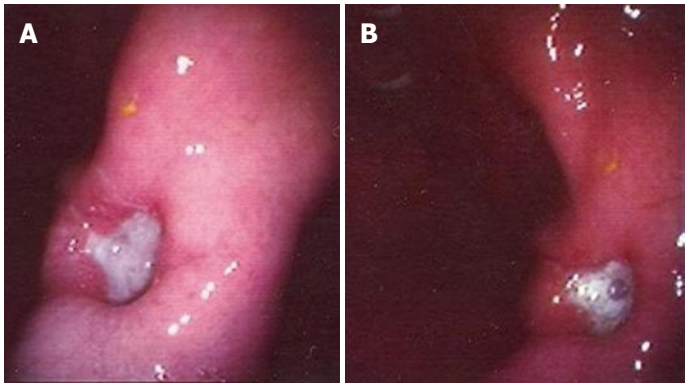


Figure 2 (**A** & **B**) Upper GI endoscopy of the patient, showing a large ulcerative lesion in the prepyloric region.

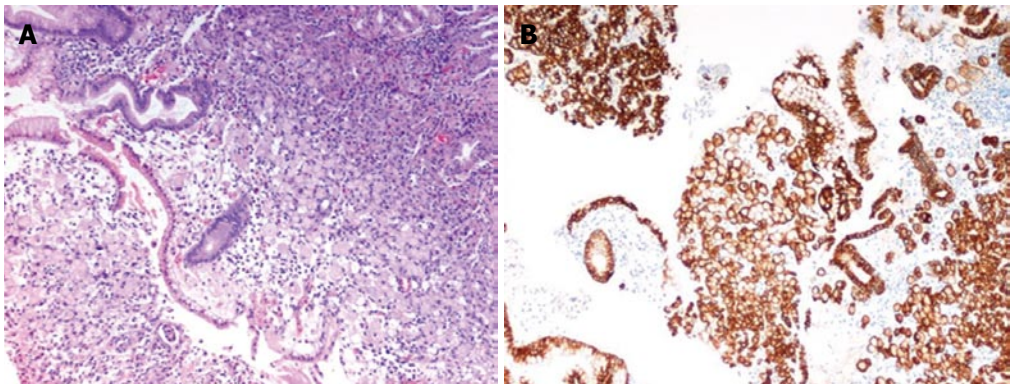


Figure 3 Gastric biopsy showing infiltration from neoplastic cells with signet-ring features (Hematoxylin & eosin, 100 ×). Right picture shows immunohistochemical stain for CK-7 (Hematoxylin & eosin, 100 ×).

primaries is unusual. Most cases are related with extensive disease burden and carry an unfavorable prognosis[5]. Breast invasion is the initial manifestation of the disease in 25%-40% of the cases[2]. Mammographically, metastatic tumors in the breast present as well-circumscribed nodules without microcalcifications, mimicking benign breast lesions[5]. Calcifications may be seen in metastatic deposits from ovarian carcinoma with psammoma bodies[6]. The most common localization of metastatic lesions is the upper-outer quadrant of the left breast. Breast involvement is bilateral in 25% of the cases, and there is concomitant axillary lymph node enlargement in up to 15%[5]. The occurrence of multiple tumor nodules is unusual and diffuse involvement mimicking inflammatory breast carcinoma is rare[7,8]. The latter clinical manifestation, due to lymphatic invasion, is rapidly progressive, and may produce clinical signs of breast involvement before a firm metastatic nodule is formed, which is even more common in hematological malignancies (leukemias, lymphomas).

Breast metastases from gastric cancer are extremely rare. To date, only 25 cases have been reported in the English speaking literature and are mentioned in Table 1. Signet-ring features have been reported in 13 cases. From reports with available data, it seems that breast involvement usually follows the detection of gastric carcinoma. Breast metastasis as initial manifestation of a primary gastric tumor, as in our case, has been reported in 10 cases. In half of these cases breast involvement had the clinical appearance of inflammatory breast cancer.

Breast metastases from gastric carcinoma differ from primary breast cancer in their histopathologic characteristics. Immunostaining is usually negative for estrogen and progesterone receptors[9,10] as well as for c-erbB-2[3]. GCDFP-15 (gross cystic disease fluid protein-15) is also absent[10]. There is no evidence of an *in situ* component[3,5] and a lack of desmoplastic response[3]. On the contrary, lymphatic tumor emboli and epithelial markers like CK7, CK20 and CEA are usually present[3,5].

In our case, the presence of signet-ring cells within breast lymphatics, the absence of mass or microcalcifications in the mammography, the lack of an *in situ* component and hormone receptor negativity strongly suggested an extra-mammary primary. It is of noted interest that Steinbrecher and Silverberg in 1976[11] first described primary signet-ring carcinoma of the breast, as a subtype of lobular carcinoma, with aggressive biological behavior and an increased tendency to metastasize in the abdomen and in the gastrointestinal tract compared to other histological subtypes[9,10]. Signet-ring carcinoma of the breast metastatic to the stomach has also been reported[12,13].

The presence of cystic ovarian masses suggested metastatic ovarian involvement (Krukenberg's tumor). Unfortunately, the patient's unfavorable clinical condition did not allow histological confirmation. Metastatic involvement of the breast and the ovaries is extremely rare and has been reported in only 5 cases so far. Selective invasion of hormone-dependent organs seems quite intriguing, especially in pre-menopausal women. Increased blood supply of the breast has been proposed as the

Table 1 Patients' characteristics[1] in reported cases of breast metastasis from gastric cancer

n	Reference[2]	Age	Histology	Presentation after initial diagnosis (mo)	Clinical presentation	Localization	Other metastatic sites
1	Reitmann 1908	33	Scirhous	-	-	R+L[3]	-
2	Kreibich 1909	65	Scirhous	-	-	R	Skin
3	Mourier 1910	31	Mucinous	-	-	L	Liver, pancreas
4	Stahr 1922	46	Anaplastic	-	-	R+L	-
5	Dawson 1936	25	Mucinous	-	-	R+L	Ovaries
6	Abrams 1949	-	-	-	-	-	-
7	Sandison 1959	56	Signet-ring cell	-	-	L	-
8	Hajdu 1972	-	Adenocarcinoma	-	-	L	-
9	Silverman 1974	-	Mucin-producing	Concomitant[4]	-	-	-
10	Toombs 1977	-	-	-	-	-	-
11	Satake 1980	39	Signet-ring cell	Concomitant	Nodule	L	-
12	Togo 1980	70	Signet-ring cell	Concomitant	Nodule	L	-
13	Nielsen 1981	59	Mucinous	Concomitant	Nodule	L	-
14	Champault 1982	65	Adenocarcinoma	Concomitant	Nodule	L	-
15	Kasuga 1986	48	Signet-ring cell	31	Nodules	R+L	-
16	Alexander 1989	28	Mucinous differentiation	Concomitant	Nodules	R+L	Lymph nodes
17	Hamby 1991	31	Signet-ring cell	Concomitant	Nodule	R	Lymph nodes, ovaries
18	Cavazzini 1993	-	Signet-ring cell	-	Inflammatory	-	-
19	Domanski 1996	48	Signet-ring cell	Concomitant	Nodule	L upper-outer	L supraclavicular
20	de la Cruz Mera 1998	61	Signet-ring cell	13	Nodule	L upper-outer	Pleura
21	Briest 1999	46	Signet-ring cell	2	Inflammatory	R+L	Bilateral axillary nodes
22	Kwak 2000	41	Signet-ring cell	Concomitant	Inflammatory	L	Ovaries[5]
23	Kwak 2000	23	Signet-ring cell	Concomitant	Inflammatory	R	Axillary nodes
24	Madan 2002	39	Signet-ring cell	3	Nodule	R	Ovaries, peritoneum
25	Di Cosimo 2003	39	Signet-ring cell	1	Nodules	R+L	Ovaries, peritoneum, skin
26	Boutis 2005	37	Signet-ring cell	Concomitant	Inflammatory	L	Ovaries[5], ascites

[1] Clinical information is mentioned in the table only if available by the authors.
[2] References Reitmann 1908, Kreibich 1909, Mourier 1910, Stahr 1922, Dawson 1936, Satake 1980, Togo 1980 are included in Hamby 1991[2].
[3] R: right; L: left.
[4] Breast metastasis was the initial manifestation of disease.
[5] No histological confirmation.

mechanism for the increased incidence of breast metastasis in pre-menopausal women[14-15]. On the other hand, gastric cancer seems to have a more aggressive biologic behavior in younger age groups, where hormonal factors are implicated[16]. The appearance of breast metastases in men with gynecomastia supports the latter hypothesis[17]. In our patient, hormonal therapy for the induction of ovulation may have influenced the natural history of the disease[1]. However, no conclusions about the pathogenesis of the phenomenon can be drawn.

Metastatic disease of the breast is rare, and usually has atypical clinical and mammographic presentation. In cases of breast inflammation or lumps, biopsy should be performed, even in the presence of an extra-mammary neoplasm. Likewise, peculiar histological features from malignant breast lesions should be co-estimated with other clinical parameters toward the diagnosis of metastatic disease. Thus, avoidance of unnecessary radical surgical procedures and an adequate treatment is more probable to be given in these patients.

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CASE REPORT

Multiple pyogenic liver abscess

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Abstract

Multiple pyogenic liver abscesses have been rarely described. We report a fatal case of multiple pyogenic liver abscesses affecting a 38-year-old woman requiring surgical drainage. Evolution was marked by occurrence of a septic shock with multi-organ system failure. The patient died 48 h after surgery. Causes, therapeutics and outcome of the disease are discussed.

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Key words: Liver abscess; Septic shock; Outcome

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INTRODUCTION

The mortality rate of pyogenic liver abscess is 11%-31%[1]. The overall mortality is high in patients with multiple liver abscesses[2]. However, multiple pyogenic liver abscesses are not frequently reported in the literature. We report a fatal case of multiple and gigantic liver abscess affecting a 38-year-old woman.

CASE REPORT

A 38-year-old woman was admitted to our hospital for abdominal pain in the right upper quadrant, fever, vomiting and anorexia as well as chills and jaundice. On examination she had marked tenderness in the right upper quadrant. The temperature was 40.1 ℃, the pulse was 119, and the respiratory rate was 30/min. The blood pressure was 80/30 mmHg. The patient did not drink alcohol.

Laboratory tests performed on admission revealed 41.5% hematocrit, 26 300 white-cell count (per mm^3), 62 000 platelet count (per mm^3), 30% prothrombin time, 269 μmol/L total bilirubin, 170 μmol/L conjugated bilirubin, 11 mmol/L urea nitrogen, 42 μmol/L creatinine, 4.5 mmol/L glucose, 117 mmol/L sodium, 4 mmol/L potassium, 87mmol/L chloride, 16 mmol/L carbon dioxide, 51U/L aspartate aminotransferase, 21 U/L alanine aminotransferase and 208 U/L creatine kinase.

Ultrasonography of the abdomen revealed that the hepatic parenchyma appeared diffusely heterogeneous, with hypoechoic foci in the right lobe, suggesting the presence of abscess or multiple hydatid cysts.

CT scan of the abdomen (Figures 1A-B) before and after intravenous injection of contrast material confirmed the presence of multiple irregular abscesses varying in size, predominantly in the right lobe of the liver.

Microbiological culture was performed (hemoculture) and the patient received intravenously cefotaxime, gentamicin, metronidazole, omeprazole, fluid, and electrolytes. Surgical drainage was performed 48 h after hospital admission. *Escherichia coli, Bacteroides fragilis and prevotella oralis* were found in cultures of the pus.

Evolution was marked by occurrence of septic shock with multi-organ system failure. The patient died 48 h after surgery.

DISCUSSION

Pyogenic liver abscesses are found in 0.3%-1.4% of autopsies[3]. Patients with diabetes mellitus, immune deficiency, sickle cell anemia, malignancy, and liver transplants are at a greater risk for developing liver abscess[1]. The majority of pyogenic liver abscesses are caused by infection originating in the biliary or intestinal tracts[4]. About 10% of pyogenic liver abscesses develop as a result of bacteria entering the liver via the hepatic artery[5]. In the majority of cases, more than one organism has been isolated from their abscesses[6]. The most commonly infecting organisms are Gram-negative aerobes and *Escherichia coli* is the most frequently encountered[7]. In our case three organisms were identified, suggesting that pyogenic liver abscesses are caused by infection originating in the intestinal tract. Without appropriate diagnosis and treatment, pyogenic liver abscesses are almost uniformly fatal[2,4]. Early diagnosis as well as treatment with appropriate antibiotics and selective drainage can substantially reduce mortality. Our observation has confirmed this hypothesis.

Antibiotic therapy should include penicillin, which is

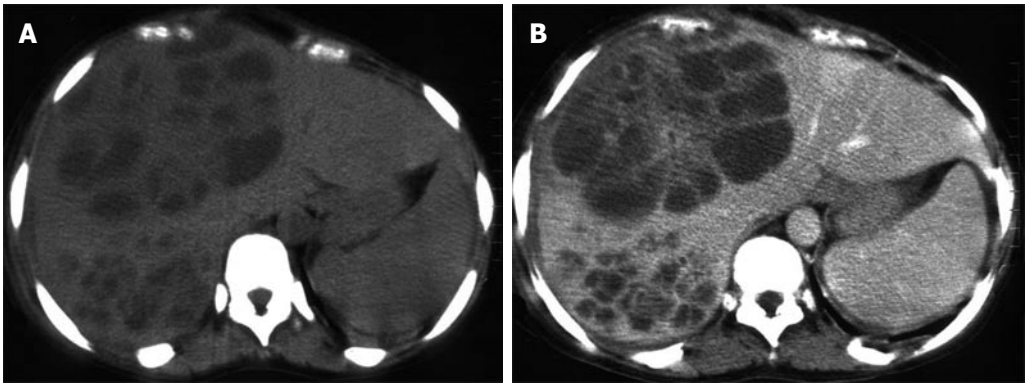


Figure 1 CT scan without (**A**) and with (**B**) contrast injection of the abdomen showing multiple irregular liver abscesses in the right hepatic lobe.

effective against *E coli, K pneumonia, bacteroides, enterococcus,* and *anaerobic streptococci*[1]. Antibiotics alone are effective for only a few patients and most patients require percutaneous aspiration or catheter drainage guided by ultrasonography or computed tomography[1]. In our case open surgery was performed because the patient suffered from multiple liver abscesses and septic shock.

The overall mortality may be as high as 30%-40% in patients with multiple liver abscesses, malignant biliary obstruction, inadequate drainage, and immunodeficiency[1,2]. In our case no pathological antecedent was found, however we cannot exclude immunodeficiency in this woman.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
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Edinburgh Convention Bureau
convention@edinburgh.org
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
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www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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EDITORIAL

Current and future applications of magnetic resonance imaging and spectroscopy of the brain in hepatic encephalopathy

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Abstract

Hepatic encephalopathy (HE) is a common neuropsychiatric abnormality, which complicates the course of patients with liver disease and results from hepatocellular failure and/or portosystemic shunting. The manifestations of HE are widely variable and involve a spectrum from mild subclinical disturbance to deep coma. Research interest has focused on the role of circulating gut-derived toxins, particularly ammonia, the development of brain swelling and changes in cerebral neurotransmitter systems that lead to global CNS depression and disordered function. Until recently the direct investigation of cerebral function has been difficult in man. However, new magnetic resonance imaging (MRI) techniques provide a non-invasive means of assessment of changes in brain volume (coregistered MRI) and impaired brain function (fMRI), while proton magnetic resonance spectroscopy (^{1}H MRS) detects changes in brain biochemistry, including direct measurement of cerebral osmolytes, such as myoinositol, glutamate and glutamine which govern processes intrinsic to cellular homeostasis, including the accumulation of intracellular water. The concentrations of these intracellular osmolytes alter with hyperammonaemia. MRS-detected metabolite abnormalities correlate with the severity of neuropsychiatric impairment and since MR spectra return towards normal after treatment, the technique may be of use in objective patient monitoring and in assessing the effectiveness of various treatment regimens.

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Key words: Hepatic encephalopathy; Magnetic resonance imaging; Magnetic resonance spectroscopy; Diffusion weighted imaging; Arterial spin labeling; Functional MRI

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INTRODUCTION

Although the phenomenon of mental disturbance in patients with disordered liver function was probably first described over 2000 years ago by Hippocrates, the precise mechanisms associated with the development of hepatic encephalopathy (HE) remain unclear. The syndrome occurs in two distinct forms: in the context of acute liver failure, acute HE results in impaired consciousness which may progress to coma and death over a period of hours or days, whereas chronic HE describes the neuropsychiatric syndrome, most commonly associated with hepatic dysfunction and portosystemic shunting in cirrhosis. This may also exist in patients with surgical portosystemic shunts[1]. In most patients the condition is "minimal" and, on a functional basis, it is discernible in prolongation of reaction times in the activities of daily living, such as driving and operating machinery, but in about 30% of patients with chronic liver disease, the syndrome is more severe with alterations in behavior, mood, personality and disturbances in consciousness[1].

In routine clinical practice, brain imaging in the form of a CT scan has been commonly used to exclude other

diagnoses, such as subdural hematomata, which may also cause impaired consciousness in patients with chronic liver disease. Until recently, the use of other imaging modalities such as magnetic resonance imaging (MRI) has been a second-line investigation in many countries, whereas magnetic resonance spectroscopy (MRS) has been largely confined to research applications, where significant advances in the understanding of pathophysiological mechanisms have been made. While most clinical MRI systems have operated at magnetic field strengths of 0.5-1.5 Tesla (T), higher field strength whole body systems are now increasingly becoming available at 3T and above, providing increased resolution and sensitivity. This article will review the published MR studies in HE and with the advent of new MR imaging capabilities, we speculate on areas of new research for the future.

HEPATIC ENCEPHALOPATHY

"Minimal" HE is the term used to describe the presence of cognitive impairment on psychometric testing and/or slowing of electroencephalographic (EEG) mean cycle frequency in the absence of any clinically overt signs[1,2]. This form of HE is present in the majority of patients with cirrhosis, but where the condition is more profound, it is usually characterised by remissions and relapses, manifested by obvious mood disorders, personality changes and disturbances in consciousness[1]. However, in a small minority of patients the neuropsychiatric impairment may follow a more persistent course. Elevated circulating ammonia levels, which are present owing to impaired hepatic nitrogen excretion or because of portosystemic shunting, are thought to be key to the changes in neurotransmitter systems that underlie the disturbances in function observed clinically[3]. These include increased GABAergic tone, elevated glutamine levels and changes in several other neurotransmitter systems and their receptors, such as reduced glutamate excitatory transmission and increased serotonergic inhibition[4]. Other abnormalities include selective alterations of blood-brain barrier permeability and changes in cerebral energy metabolism[5]. However, the central question of how these relate to elevated ammonia concentrations is an unresolved issue.

MAGNETIC RESONANCE IMAGING

CT and MRI have long been used to detect obvious structural abnormalities in the brain such as cerebral atrophy, which occur in patients with chronic alcoholism, who do not necessarily have significant liver disease[6,7]. However, MR techniques may provide non-invasive insight into the pathogenesis of HE. Apart from structural changes associated with the direct effects of alcohol, a longstanding MR observation has been that patients with cirrhosis of any aetiology show hyperintensity in the basal ganglia on T_1-weighted MRI (Figure 1)[8-11]. Hypermanganesemia due to occupational exposure or total parenteral nutrition also results in similar appearances, which reverse after correction of the blood manganese levels[12,13]. In cirrhosis, a correlation between blood manganese levels and globus pallidus signal intensity has been reported[10,14,15] and elevated *post-mortem* globus pallidus manganese concentrations have been detected in patients who have died with terminal liver disease[10,16]. It was initially suggested that basal ganglia hyperintensity might be an important aetiological association of the form of HE that complicates chronic liver disease[8,11]. However, a number of studies found no correlation between basal ganglia T_1-weighted signal intensity and clinical encephalopathy or neuropsychological test performance[9,17,18], although the extrapyramidal symptoms seen in a minority of patients with HE may be related[14]. Instead, basal ganglia T_1-weighted signal intensity and manganese accumulation appears to be related to the underlying degree of hepatic dysfunction or more particularly to the severity of cholestasis and/or the presence of portosystemic shunting, rather than directly to neuropsychiatric impairment[19]. The relationship with cholestasis is important, since manganese itself is normally eliminated from the body via the biliary route[20].

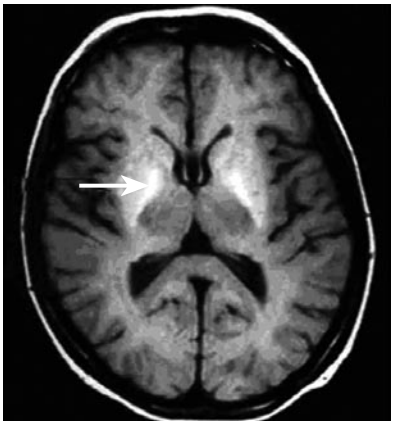


Figure 1 T1-weighted brain MR image through the basal ganglia acquired at 1.5T, showing bilateral globus pallidus (arrow) hyperintensity in a patient with cirrhosis of the liver.

It has recently been proposed by Häussinger and colleagues that a major contributory event in the development of HE in patients with chronic liver disease is an increase in astrocyte hydration, or in other words, a low-grade cerebral oedema without a clinically overt increase in intracranial pressure[5]. This may occur as a consequence of ammonia uptake by astrocytes and subsequent detoxification by the enzyme glutamine synthetase to form glutamine from glutamate[3]. This deamidation process is thought to result in the accumulation of glutamine within astrocytes, which acts as a cerebral osmolyte with resultant cell swelling. With conventional neuroimaging, brain oedema is not evident in the vast majority of patients with chronic liver disease who have HE, probably because if present, the brain swelling is minimal and is thus beyond the threshold for detection on standard MRI. This is unlike the encephalopathy associated with fulminant hepatic failure, which is obviously characterised by cerebral oedema [21]. However, a number of recent studies, which employed magnetization transfer imaging (MT), suggested that there may be an increase in total brain water in the HE of chronic liver disease[22-24].

MAGNETIZATION TRANSFER IMAGING

MT allows indirect measurement of bound and free

water compartments in the brain and may be affected by changes in membrane fluidity, heavy metal deposition and alterations in total water content[23,24]. The technique itself is a method for manipulating tissue contrast[25,26]. In addition to enabling the acquisition of enhanced contrast images, techniques employing MT also allow the measurement of MT ratios (MTR). The MTR is a quantitative tissue characteristic that reflects the behavior of normally MR-invisible protons bound to macromolecules. MTR measurement may detect brain parenchymal changes that may not be visible using standard MR techniques. In essence, protons in tissues can be described as existing in two pools. The free pool consists of mobile protons, such as those in body water, and has a narrow spectral line with relatively long T_1 and T_2 relaxation times (Figure 2). The bulk of the signal in conventional MR systems comes from this free pool, as the MR excitation frequency range is narrow and centered upon these freely mobile protons. A second pool of protons bound in proteins, other macromolecules and membranes, is conventionally said to be MR invisible, as it falls outside the typical excitation range. This pool has a broad spectral line and very short relaxation times (Figure 2). Magnetization can be transferred bi-directionally between pools by direct interaction between spins, the transfer of nuclei or by direct chemical means. Under normal circumstances, the transfer of magnetization is the same in both directions.

Techniques that employ MT do so by saturating the magnetization in the bound pool while leaving the free pool largely unaffected. This is possible because the bound pool has a broad spectral line and can be excited by the application of an "off-resonance" RF pulse (Figure 2). This saturation results in substantial attenuation of the magnetization in the bound pool. As a consequence, there is little transfer of magnetization to the free pool and both the effective longitudinal magnetization within it and its T_1 are reduced. Pulse sequences that incorporate the application of an "off-resonance" pulse can be designed to allow quantification of the effect of MT in different tissues, in health and disease[25]. Reduced MTRs may result from pathologies that alter the structural integrity and the relative macromolecular-water composition of brain parenchyma, such as in multiple sclerosis plaques or in end-stage cirrhosis[27-29]. These observations may also result from the deposition of paramagnetic substances in the brain. MR experiments with manganese chloride phantoms have demonstrated a strong inverse linear relationship between the manganese concentration and the MTR[29]. This property has been exploited for the use of paramagnetic contrast agents, such as gadolinium-based compounds. Furthermore, manganese is excreted via the biliary route and can thus accumulate in cholestatic conditions, including in the basal ganglia in patients with chronic liver disease[19]. It is of note, therefore, that our group has reported reduced MTRs in the basal ganglia of fatigued patients with primary biliary cirrhosis, which correlate strongly with ambient plasma manganese levels[30].

In the context of HE, the reported change in MTR in patients with cirrhosis could represent an alteration in the structure of cell membranes, leading to cell membrane fluidity. However, these observations may, perhaps, be accounted for by an increase in cerebral water content[22-24]. If the interpretation of the MT papers in HE is correct[22-24], an increase in brain hydration, as represented by altered MT ratios, will not be dramatic enough to cause an increase in intracranial pressure, but may reflect "lower-level" astrocyte swelling, which could be sufficient enough to trigger alterations in astrocyte function[22-24]. The possible functional effects of low grade astrocytic swelling are numerous and could possibly explain many of the features of HE complicating chronic liver disease, including alterations in blood brain barrier permeability, neurotransmitter processing and receptor density and upregulation of cerebral neurosteroid production[5]. At present, the MTR data remain an interesting observation and future studies incorporating newer MR imaging techniques are required to provide more definitive answers on pathogenesis.

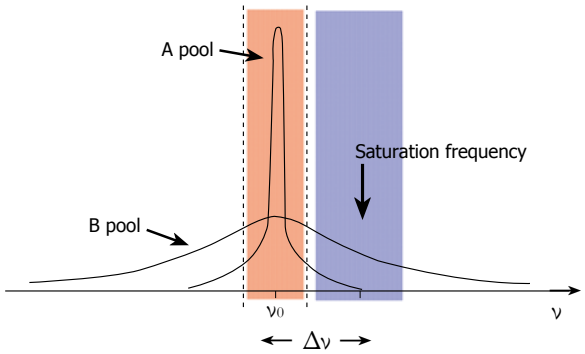


Figure 2 Model demonstrating the concepts underlying the phenomenon of magnetization transfer. The free pool of protons (**A**) resonates at the Larmor frequency (ν_0) and has a narrow spectral line. It is excited by an RF pulse which covers the frequencies shown in pink. The "bound" pool of protons (**B**) has a broad spectral line and can be excited and saturated by the application of RF irradiation at a frequency offset by $\Delta\nu$, shown in blue, without significantly affecting pool A.

DIFFUSION IMAGING

Diffusion-weighted imaging (DWI) is a method used to quantify the movement of water molecules. One of the first applications of this technique was the detection of acute cerebral ischemia in the early 1990s[31,32]. Further recognized indications include the investigation of multiple sclerosis[33] and brain tumors[34,35].

The diffusion of water molecules follows the principles of Brownian motion. Thus, when these molecules are unconstrained, their movement is random and therefore equal in all directions, being described as "isotropic". However, the motion of water molecules in structured environments is restricted, according to the physical surroundings. The motion of water molecules in the brain is restricted by the microstructure within grey and white matter. Water molecules on average tend to move parallel to, rather than perpendicular to the white matter tracts[36,37]. This motion is described as "anisotropic", as it is no longer equal in all directions. The motion of the molecules in the x, y and z directions and the correlation between

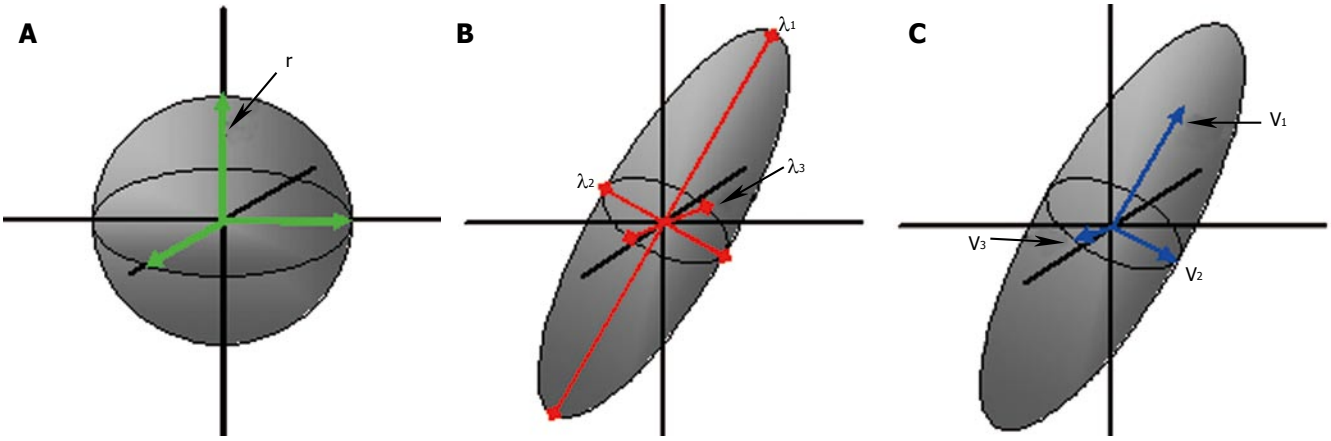


Figure 3 Principles of diffusion: A water molecule in an unconstrained environment would move randomly and equally in all directions; isotropic diffusion (**A**). The probability of motion can be defined by the radius '*r*', of the spherical range of motion. Diffusion in an ordered environment such as the white matter will assume an elliptical range of motion (**B** and **C**). Three eigenvalues, λ_1, λ_2 and λ_3 define the shape and three eigenvectors v_1, v_2 and v_3 define the orientation of the ellipsoid.

the directions is described with the diffusion tensor[38,39]. A tensor is a mathematical construct that defines the properties of a three-dimensional ellipsoid. To define the diffusion tensor requires acquisition of diffusion data in a minimum of six non-collinear directions, this is known as diffusion tensor imaging (DTI). The diffusion tensor is graphically represented by a three-dimensional ellipsoid, with the long axis representing the primary direction of motion (Figure 3). Eigenvalues and eigenvectors describe the magnitude and directions of the three principal axes of the diffusion ellipsoid[40]. The diffusion tensor is evaluated at each pixel location and so maps of the magnitude and dominant direction of diffusion can be produced. The dominant directions can be followed from one pixel to another, plotting lines along which diffusion is likely to occur. This practice is known as tractography, the name coming from the implication that these paths are believed to represent white matter tracts. Using a minimum of 2 differently weighted DWI images, a measure of diffusion can be calculated. These data can then be mapped to create an apparent diffusion coefficient (ADC) image[41]. The ADC is a measure of tissue water diffusivity and is dependent upon interactions between water molecules and their surrounding chemical and structural environment[42]. Diffusion data may be expressed in terms of the mean diffusivity, sometimes called the mean ADC or the trace ADC. This is the average of three ADC measurements in three orthogonal directions. DTI gives rise to detailed information on the microstructure within an imaging voxel, including mean diffusivity, the degree of anisotropy and the direction of diffusivities[43]. The mean diffusivity reflects the displacement of water molecules and the presence of obstacles at both the cellular and subcellular level. Fractional anisotropy (FA) and relative anisotropy (RA) are commonly used to express the degree of anisotropy. Anisotropy will be related to the characteristics of any physical barriers, such as the orientation, density, size and shape of nerve fibers within white matter tracts. However, interestingly, myelination has been demonstrated not to be a primary determinant of anisotropy, but may indeed have modulatory effects[44]. The directionality of

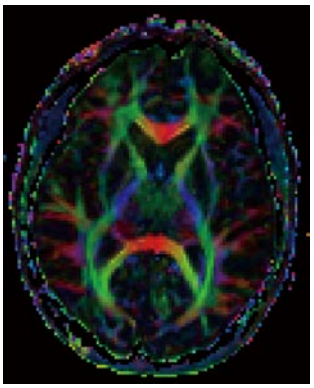


Figure 4 Color fractional anisotropy (FA) image from a 44 year-old healthy male volunteer. Diffusion tensor imaging (DTI) performed on a 3T Philips Intera™ using 32 different directions of diffusion sensitization. The different colors represent the principle diffusion directions and hence the direction of the white matter tracts: green represents anterior-posterior, blue represents caudo-cranial, red represents transverse. FA maps were generated using DTI studio version 2.1©, (Jiang and Mori, Johns Hopkins University, MD, USA).

fibers can be expressed on color-coded two-dimensional maps, or by three-dimensional tractography images. The orientation of the major axonal fiber bundles can be calculated using the eigenvectors and eigenvalues by various algorithms[40]. The expression of DTI data in the form of 3D images is one of the latest developments in this technique and may provide insight into defects in brain connectivity (Figure 4).

In the field of HE, one study has looked at regional brain ADC values in patients with cirrhosis, secondary to viral hepatitis[45]. Seven patients had no encephalopathy, six had grade I and one grade II hepatic encephalopathy, according to Parsons-Smith criteria[46]. The authors found that except in the thalamus, ADC values were significantly increased in all white matter and deep grey matter areas examined. Furthermore, except for the caudate nucleus, there was a positive correlation between ADC values in all areas of the brain and venous ammonia concentrations. They suggest that their findings are related to increased brain water content, which would support the brain swelling hypothesis.

FUNCTIONAL MRI

It is well recognized that HE encompasses a broad spectrum of neuropsychiatric and cognitive impairments. Investigators have used a variety of non-invasive

techniques, such as electroencephalography (EEG)[47] and magnetoencephalography (MEG) [48] to study the brains of patients with chronic liver disease, but these methodologies suffer from limited spatial resolution. Positron emission tomography (PET) has also been used to investigate both metabolic impairments and neurotransmitter deficits in patients with HE[49-52], but PET does not readily lend itself to serial studies, owing to the necessity for injection of radioactive tracers.

However, functional MRI (fMRI) provides the opportunity to study brain function in detail without the drawbacks of those other techniques, albeit with a reduced sensitivity resulting in long scan times. The most common method of assessing an alteration in cerebral activity in fMRI is by measuring the "blood oxygenation level dependent" (BOLD) signal, which represents local changes in hemoglobin oxygenation due to cerebral blood flow changes[53-55]. The signal arises because of the different magnetic properties of oxygenated hemoglobin, which is isomagnetic, and deoxyhemoglobin, which is paramagnetic relative to tissue[56]. PET studies have previously demonstrated that cerebral blood flow increases by up to 50% in response to functional activation, but the cerebral metabolic rate for oxygen increases only by 5%[57,58]. Thus, in fMRI there is a reduction in the proportion of deoxyhemoglobin, and hence in the magnetic properties of the activated brain region, which contributes to the BOLD signal. In the context of chronic liver disease, one study has used fMRI to investigate cognitive alterations in a cohort of 9 patients with minimal or "non-manifest" hepatic encephalopathy (nmHE)[59]. The patients were given a stimulus of a flickering light of decreasing frequency and were asked to indicate when the light appeared to change from a fused light to a flickering light; the "critical flicker frequency" threshold[60]. The BOLD signal was measured during this task, and the investigators observed a decrease in activation in the right inferior parietal cortex of the nmHE patients, compared to healthy age-matched volunteers. These data also suggested impaired neural connections between several cortical structures[59].

CEREBRAL BLOOD FLOW

It is well known that cerebral blood flow (CBF) changes in encephalopathic patients[49,61]. There are a variety of MRI techniques that can potentially be used to study this, including arterial spin-labeling (ASL) and contrast-enhanced perfusion MRI (CEMRI)[62-64]. Perfusion can be calculated by measuring the change in signal in the brain as a contrast agent (Gd-DTPA) flows into the brain. If an accurate measure of the arterial input function (AIF) can be extracted then quantitative measures of blood volume, flow and transit time can be calculated. Accurate measurement of the AIF is difficult and so absolute quantification is rare. In addition, the use of contrast agents is not desirable in patients with poor liver function. As always with contrast agents, longitudinal studies are also not recommended which explains the lack of such studies to date. An emerging methodology is arterial spin labeling where the blood is used as an endogenous contrast agent. The blood is tagged (usually in the carotid artery) using an RF pulse, similar to those used in standard MR imaging. The arrival of this "bolus" of tagged blood is then monitored in the brain by imaging. ASL is a very low sensitivity method compared to CEMRI and so requires significantly longer imaging times to acquire robust data (10-20 min compared to < 1 min for CEMRI). This means that ASL really only becomes feasible at higher field strengths (3T+), where imaging times become acceptable due to increased sensitivity. At the time of writing this methodology has yet to be applied to the study of HE, but the technology is now at a level of development that the known CBF changes associated with HE may be able to be quantified and response to treatment tracked using such methods.

VOLUMETRIC MRI

Until recently, MR measurement of brain and ventricular volumes was not sensitive enough for direct, serial measurement of small changes in brain size in patients with HE, who have structurally normal brains. However, the rapid development of MR analysis software now allows for precise measurements of whole or regional brain volumes and allows very small differences in brain size to be monitored that may not be visible to standard radiological inspection (coregistered MRI)[65,66]. These volume changes may be measured to allow inter- or intra-group comparisons and to follow up individuals or groups longitudinally. The techniques have been applied to a number of clinical conundra including pre-eclampsia[67-70] and given the recent interest in the possibility of low-grade oedema as a central factor in chronic HE[5], a useful application of MR is to investigate the brain volume changes that may occur in HE. One such study was performed by our group[71]. Six patients had minimal HE and three had overt HE. Seven patients were treated with lactulose for 6 weeks and three of them improved on serial psychometric testing with a consequent reduction in brain size on repeat MR imaging. Although measurable changes were found after treatment intervention, these were not correlated with the degree of encephalopathy. A recent study used voxel-based morphometric analysis of MR data to calculate brain volumes in patients with cirrhosis of varying etiologies[72]. It is not clear whether these patients were formally assessed for the presence of encephalopathy. However, the investigators found that patients with alcoholic cirrhosis had atrophy involving both grey and white matter structures[72], the latter finding in white matter is consistent with early neuropathological studies[73]. Interestingly these authors also found that patients with cirrhosis, caused by viral hepatitis also had similar, but quantitatively less atrophy[72].

LOCALIZED BRAIN WATER CONTENT

One imaging group has developed an MR technique to measure localized cerebral water content in a quantitative fashion[74,75]. This technique has been used to investigate

a cohort of patients with hepatic encephalopathy[76]. Results from this pilot study show a significant correlation between the severity of HE and white matter water content. This could be a technique of great interest, if it can be translated to other MR systems in other centers around the world.

MAGNETIC RESONANCE SPECTROSCOPY

Further evidence for changes in brain hydration and cerebral osmolytes in HE which complicates chronic liver disease comes from studies using MRS. *In vivo* MRS is a non-invasive technique which can be used to provide localized biochemical information on cerebral metabolic processes. The underlying principles of physics that govern MRS are essentially the same as for MRI. Nearly all elements possess at least one naturally occurring isotope amenable to MR study. The most commonly used isotopes have been hydrogen-1 (^{1}H), and phosphorus-31 (^{31}P). ^{1}H MRS can be utilized to provide information on brain metabolites such as choline, creatine, N-acetyl aspartate (NAA), glutamine and glutamate, as well as osmolytes such as myoinositol and taurine[77].

Several studies have reported characteristic spectral appearances in HE, typified by a reduction in the myoinositol and choline resonances and an increase in the glutamate/glutamine composite resonance, which correlate with neuropsychiatric impairment (Figure 5)[77-85]. Ammonia itself cannot be detected by ^{1}H MRS, but the increase in signal from the glutamine/glutamate is likely to reflect ammonia incorporation into glutamine. The astrocyte swelling hypothesis predicts that homeostatic mechanisms occur to limit the osmotic load in astrocytes, leading to the extracellular release of osmolytes, such as myoinositol[5]. This is supported by the observation of reduced intracellular myoinositol in ^{1}H MRS studies.

The choline resonance contains contributions from phosphocholine and glycerophosphorylcholine, which are cell membrane precursor and degradation products[86]. This observed reduction is likely to reflect alterations in phospholipid metabolism, membrane fluidity, or secondary changes in water content, because glycerophosphorylcholine is itself a cerebral osmolyte[87].

The typical cerebral ^{31}P MR spectrum contains at least seven resonances, which can be assigned to phosphomonoesters (PME), inorganic phosphate (Pi), phosphodiesters (PDE), phosphocreatine, γATP, αATP and βATP (Figure 6)[88]. ^{31}P MRS provides information on the status of energy phosphates and on phospholipid metabolism. Compared to ^{1}H MRS, there is less agreement in the published cerebral ^{31}P findings in patients with HE, due to the small populations studied and variation in the techniques that have been used. Early studies reported reductions in cerebral inorganic phosphate or phosphocreatine, which were interpreted as indicating defects in cerebral energy metabolism[89]. Our group has consistently found reductions in the PME/βATP and PDE/βATP ratios, which correlate with the reduction in choline using ^{1}H MRS[83,84,90,91]. A recent study which used proton-decoupled ^{31}P MRS, a technique which allows the components of the composite PDE peak to be resolved, showed a reduction in glycerophosphorylcholine, which is a major component of PDE and, as discussed above, may be reduced as a consequence of disturbances of cerebral osmoregulation[87].

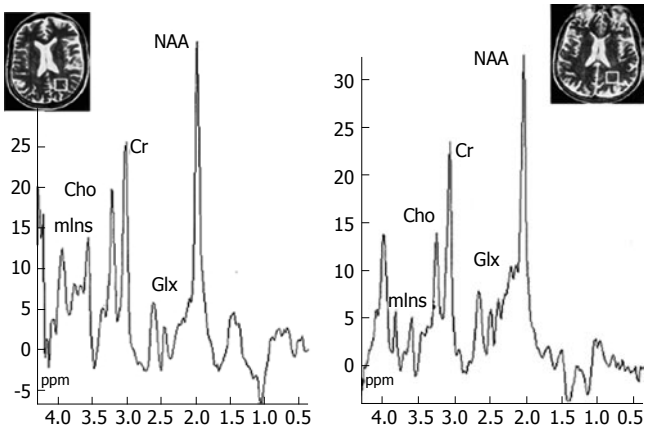


Figure 5 ^{1}H MR spectroscopy water-suppressed proton spectra of an 8 mL voxel located in the parietal region including predominantly normal appearing white matter (insert images show position of the voxel) in a healthy control subject (left) and in a cirrhotic patient (right), acquired using a STEAM pulse sequence [1600/20/30/256 (TR/TE/TM/acquisitions)]. The main resonances correspond to N-acetylaspartate (NAA, 2.0 ppm), glutamate/glutamine (Glx, 2.1-2.5 ppm), creatine/phosphocreatine (Cr, 3.02 ppm), choline-containing compounds (Cho, 3.2 ppm), and myo-Inositol (mIns, 3.55 ppm). Comparison of the spectra shows a decrease in Cho and mIns resonances with an increase in the glutamate/glutamine region in the cirrhotic patient. Reproduced with the permission of Springer Science and Business Media, from Cordoba *et al* 2002[28].

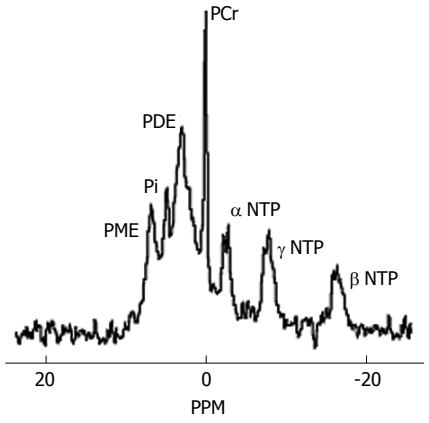


Figure 6 Normal brain phosphorus-31 MR spectrum acquired at 1.5T. PME = phosphomonoesters, Pi = inorganic phosphate, PDE = phosphodiesters, PCr = phosphocreatine, NTP = nucleotide triphosphate. The PME resonance provides information on cell membrane precursors and the PDE resonance on cell membrane degradation products.

FUTURE MRS DEVELOPMENTS

The current limitations of MRS include the limited resolution of metabolites which resonate within a narrow spectral range, such as glutamine and glutamate, and the inability to detect low concentration metabolites. Possible advances in the field include the use of two-dimensional spectroscopy, spectral editing and the use of higher magnetic field strength systems (for example at 3T).

Of these, two-dimensional spectroscopy has already been used to investigate HE. Investigators have reported enhanced spectral resolution with the additional finding of an increased taurine resonance, which was well resolved [92] (Figure 7). However, currently this technique suffers from

low signal-to-noise, and despite the use of a surface head coil, it is unable to probe the deeper grey matter structures, such as the basal ganglia.

Spectral editing involves only the acquisition of a desired spectrum of interest by suppressing the signal of other metabolites and then attempting to acquire only the signal of interest. This approach is of importance in HE in order to separate glutamate from glutamine, which overlap in conventional MR spectra. It should be noted that spectral editing is particularly sensitive to motion artefact and instrument instability. However, these methodologies have been used to highlight taurine, GABA and myo-inositol *in vivo* and suppress unwanted resonances[93-95]. Such sequence development promises much in the future for HE patients, but with respect to resolving glutamate from glutamine *in vivo*, another approach called averaged TE spectroscopy has been used to resolve glutamate from glutamine[96]. This has been used in multiple sclerosis at 3T, but not yet in HE patients[97].

With the advent of clinical magnet systems operating at higher magnetic fields, such as 3T, which is twice that used hitherto in published studies, the sensitivity of the technique may be enhanced further with improved signal-to-noise ratios. The possibility of performing multiple MR sequences in the same examination of the brain is now possible, because acquisition times are usually faster at these higher field strengths. It will thus be feasible to perform MRS studies on cerebral osmolyte levels in the same examination as acquiring data suitable for computer-enhanced visual detection of brain swelling and fMRI which should pinpoint cognitive and perceptive derangements. Since MR does not involve ionising radiation, serial studies will be possible to look at treatment effects in HE.

CONCLUSIONS

In vivo MRI and MRS provide a non-invasive way of studying brain chemistry and metabolism in patients with liver disease. These techniques have been central in the study of the underlying mechanisms of liver-related brain disease. MRS remains a research tool in the setting of liver disease. However, simple MRS sequences can be added to a standard MR examination to provide metabolic information that could help monitor response to therapy. MR studies in HE have provided important *in vivo* data, which have contributed to the current theories on astrocyte swelling[5]. This model accommodates many of the common precipitating factors for CHE, such as hyponatremia, benzodiazepine administration and infection (raised inflammatory cytokines), since these events also lead to astrocyte swelling *in vitro*[5]. Astrocyte swelling may therefore be a common pathway for multiple synergistic factors in the pathogenesis of HE complicating chronic liver disease. However, further MR studies are clearly required before these ascertations can be confirmed. A whole variety of new MR techniques now exist to study HE in more depth.

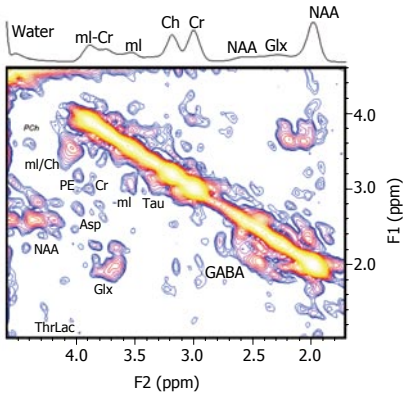


Figure 7 A localized two-dimensional correlated spectroscopy (two-dimensional L-COSY) spectrum of a 40-year-old patient. A projected one-dimensional spectrum is also shown on top of the figure. Peaks are presented in the magnitude mode. Reproduced with permission from John Wiley and Sons Inc, from Binesh N *et al* 2005[93].

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REVIEW

Cellular and molecular aspects of gastric cancer

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Abstract

Gastric cancer remains a global killer with a shifting burden from the developed to the developing world. The cancer develops along a multistage process that is defined by distinct histological and pathophysiological phases. Several genetic and epigenetic alterations mediate the transition from one stage to another and these include mutations in oncogenes, tumour suppressor genes and cell cycle and mismatch repair genes. The most significant advance in the fight against gastric caner came with the recognition of the role of *Helicobacter pylori (H pylori)* as the most important acquired aetiological agent for this cancer. Recent work has focussed on elucidating the complex host/microbial interactions that underlie the neoplastic process. There is now considerable insight into the pathogenesis of this cancer and the prospect of preventing and eradicating the disease has become a reality. Perhaps more importantly, the study of *H pylori*-induced gastric carcinogenesis offers a paradigm for understanding more complex human cancers. In this review, we examine the molecular and cellular events that underlie *H pylori*-induced gastric cancer.

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Key words: Gastric cancer; *Helicobacter pylori*; Host-microbial interactions; Inflammation; Molecular mechanisms; Host genetics

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INTRODUCTION

Gastric cancer remains a major health problem being the fourth commonest cause of cancer death in Europe[1]. On a global scale, gastric cancer remains the world's second commonest malignancy, having only been overtaken by lung cancer in the late 1980's[2-4]. There is substantial international variation in gastric cancer incidence with the highest rates reported from Korea, Japan and eastern Asia. Other high incidence areas include Eastern Europe and parts of Latin America, while Western Europe and the US generally have low incidence rates. The global burden of gastric cancer is shifting rapidly from the developed world to the developing world. Despite the worldwide decline in incidence and the major improvements in diagnosis and treatment, less than 20% of patients survive to 5 years.

PATHOLOGY OF GASTRIC CANCER

The vast majority of gastric cancers are sporadic. However there is strong evidence that occasional cases have an inherited component. Over 95% of malignancies of the stomach are adenocarcinomas. Lymphoma, sarcomas and carcinoid tumours comprise the remaining less common neoplasm's. Adenocarcinoma of the stomach comprises a spectrum of different conditions classified according to the site of tumour origin and the pathological appearance of the lesion. Lauren composed a histological classification that is widely applied. According to this classification neoplasms are described as intestinal or diffuse types[5]. Intestinal tumours are comprised of malignant cells that are united to form structures resembling functional glands of the gastrointestinal tract. In contrast, the less common diffuse tumour type comprises cells that lack cohesion and are no longer capable of gastric function. The evolution of intestinal tumours has been characterised as progressing through a number of sequential steps. These steps begin with gastritis which progresses to mucosal atrophy (atrophic gastritis) followed by intestinal metaplasia, dysplasia and carcinoma with subsequent metastatic dissemination. No preceding steps have been identified in the pathogenesis of diffuse carcinoma other than the obvious chronic gastritis that is the hallmark of *Helicobacter pylori* (*H pylori*) pathogenesis. Diffuse adenocarcinoma has an increased propensity for intra and transmural spread and is therefore associated with a poorer prognosis. Unfortunately the histological classification of an individual gastric adenocarcinoma is not clear-cut with a tumour often comprising a mixture of intestinal and diffuse tissue types.

AETIOLOGY OF GASTRIC CANCER

The pathogenesis of gastric cancer represents a classic example of gene-environment interactions[6,7]. For many

decades it was well established that the cancer developed along well-defined histological and pathophysiological stages. Chronic inflammation with gastric atrophy was shown to be the most important pathological entity with hypochlorhydria being the most important physiological abnormality. Diets high in food preservatives such as salts and nitrates are thought to induce gastric malignancy whereas increased consumption of food containing natural antioxidants such as fresh fruit and vegetables may slow or prevent the disease. Alcohol and smoking are also thought to contribute to the aetiology. Achlorhydria, pernicious anaemia and blood group A are also associated with a higher risk of gastric malignancy.

Hereditary factors clearly increase the risk of gastric cancer and this malignancy is part of a number of familial cancer syndromes. The most celebrated familial case of gastric cancer is that of Napoleon Bonaparte. It is well documented at autopsy that the exiled Emperor had a malignant gastric ulcer complicated by a chronic perforation and haemorrhage. His father, Charles Bonaparte, died from scirrhous carcinoma of the pylorus at the age of 39, and his grandfather, Joseph Bonaparte, also died of "suspected" gastric cancer at the age of 40. At least one of his brothers and one of his sisters also died of the same malignancy[8]. Lending support to the genetic aetiological hypothesis is the recognition that patients with hereditary nonpolyposis colon cancer and Familial Adenomatous Polyposis are at an increased risk of developing malignancy in the stomach. Following the discovery of *H pylori* it is has become apparent that the preceding aetiological factors only account for a small proportion of the cases and this review will focus primarily on *H pylori*-induced sporadic gastric cancers.

H pylori infection

The discovery of *H pylori* infection in the early eighties proved a turning point in understanding the pathogenesis of gastric cancer[9]. While the link between *H pylori* and peptic ulcer disease was established soon after successful culture of the bacterium, the association with gastric cancer lagged almost a decade before credible evidence was presented. The major reason for this delay was inability to demonstrate the presence of active infection in gastric tissue of cancer patients. A major advance in this field came with the recognition that chronic *H pylori* infection induces physiological and morphological changes within the gastric milieu that increase the risk of neoplastic transformation. It is widely accepted that chronic *H pylori* infection induces hypochlorhydria and gastric atrophy, both of which are precursors of gastric cancer (Figure 1). Presence of the infection in the final stages of this cascade is therefore not necessary for cancer to develop as irreversible damage had already occurred.

Microbiology of H pylori

The *Helicobacter* genus consists of at least 24 species found in the GI tracts of animals and humans. One of these species is *H pylori* a Gram-negative, spiral shaped, microaerophilic bacilli known to chronically infect over half the world's population[10]. It is usually acquired in childhood, and if left untreated can persist for decades within the extreme environment of the human stomach[11]. The infection can be acquired via the faecal/oral or gastric/oral routes, and if not treated with antibiotics, can persist throughout life. The organism is non-invasive, nonspore-forming, measuring approximately 3.5 × 0.5 micrometers, with 4 to 6 unipolar flagellae[12]. These flagellae are protected from damage by the acidic environment of the stomach by a sheath. This allows the bacterium to move through the mucus layer within the stomach and to reside between this layer and the gastric epithelium[13]. Eighty percent of the bacilli are free-living, however the remainder adhere tightly to the underlying cells and induce ultra-structural changes in the gastric epithelial cell[10,14].

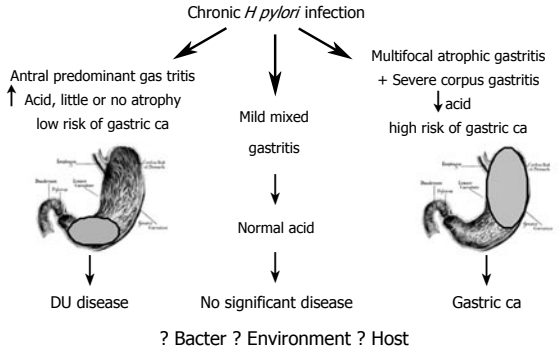


Figure 1 Divergent responses to *H pylori* infection.

Adaptation of H pylori to the gastric environment

While *H pylori* is well equipped to colonise the inhospitable acidic gastric environment, it is essentially a neutralophile that grows best at a pH of between 6.0 and 8.0[13]. In order to do this, *H pylori* is equipped with several factors that allow it to colonise and evade the host defences including the immune response. *H pylori* possesses a urease enzyme that allows it to hydrolyse gastric urea into ammonia and carbon dioxide. This permits *H pylori* to maintain a constant internal and periplasmic pH, even in the presence of a very high external H^+ concentration. In addition, *H pylori* expresses a urea transport protein (UreI) with unique acid-dependent properties that attenuates the rate of urea entry into the cytoplasm[15]. The combination of a neutral pH-optimum urease and an acid-regulated urea channel explains why *H pylori* is unique in its ability to inhabit the human stomach[16,17]. Indeed, isogenic urease-negative mutants of *H pylori* are incapable of colonising the gastric mucosa[18]. To conserve energy and resources, *H pylori* seeks out a niche that does not constantly challenge its acid-resisting and acid adaptation machinery. This explains why the initial colonisation is maximal in the antral part of the stomach, a region with a higher pH than the acid-producing corpus mucosa. This also explains why the distribution of infection changes when gastric acid secretion is inhibited by pharmacological means. In these circumstances, *H pylori* and its associated inflammation spread to involve the hitherto protected corpus mucosa.

H pylori's role in gastric disease

Since early in the 20th century, it has been known that different patterns of gastritis can occur following *H pylori* infection, which ultimately results in differing clinical outcomes. The majority of *H pylori* infected individuals develop mild pangastritis, a condition that does not adversely alter gastric physiology and is not associated with significant disease (Figure 1). Antral predominant gastritis is associated with hyperchlorhydria, which carries a low risk of developing gastric cancer, but a high risk of developing duodenal ulcer disease[19]. In contrast corpus predominant gastritis, which leads to hypochlorhydria and gastric atrophy, carries an increased risk of gastric cancer[20]. It is thought that following the development of the hypochlorhydric atrophic gastric environment, other bacteria, such as nitrogen-fixing bacteria are able to colonise. These bacteria produce carcinogenic N-nitroso compounds through the conversion of nitrates, and the increasing mutagenic and genotoxic pressure coupled with the lack of free radical scavengers is thought to drive gastric cancer progression.

The key question is how chronic *H pylori* infection can be associated with such divergent clinical outcomes? Much research has focussed on *H pylori* strain differences such as virulence factors as the source of the disease specificity. However, although these factors undoubtedly contribute to the severity of the disease, they do not define the clinical outcome[21]. This has prompted research in to other factors that could affect an individual's response to *H pylori* infection. These factors include environmental and host genetic factors.

Role of bacterial virulence factors

There is substantial evidence that genetic differences play a role in the clinical outcome of *H pylori* infection, particularly *H pylori*-virulence associated genes such as *cag*A, *vac*A, *ice*A and *bab*A.

The *cag* Island: The best characterised *H pylori* virulence factor is the *cag* pathogenicity island (*cag*-PAI), a 40 kb chromosomal DNA, which contains approximately 31 genes[22,23]. Several of the genes present on the *cag*-PAI encode components of a type IV secretion system, which allows CagA (cytotoxin-associated gene A), a 120-130 kDa protein product and other bacterial proteins encoded by the *cag*-PAI to be injected into the epithelial cell cytosol (Figure 2)[10,12]. After entering the cell, CagA is phosphorylated and binds to tyrosine phosphatase, which induces secretion of IL-8, a potent chemotactic and activating factor for neutrophils, by the activation of nuclear factor kappa B (NF-κB) complexes[10,24]. The *cag*-PAI also induces cell surface remodelling including the induction of pedestal formation, activation of the transcription factor AP-1 and expression of the proto-oncogenes c-*fos* and c-*jun* by activation of the ERK/MAP kinase cascade[25,26]. *H pylori* strains which do not contain the *cag*-PAI or possess mutated *cag* genes do not induce these changes or do so to a much lesser extent[24,26,27].

H pylori strains can be divided into 2 groups based on the presence/absence of the *cag*-PAI: - type 1 strains, which possess the *cag*-PAI, and type 2 which do not. Type 1 strains are associated with severe gastritis, peptic ulcer disease, gastric atrophy and non-cardia gastric cancer, thus linking the presence of PAI to increased virulence[28-30]. It should be noted however, that not all type 1 isolates contain the entire PAI. Infection with a cagA expressing strain is also associated with reduced apoptosis whereas infection with a cagA negative strain is associated with increased apoptosis. Therefore cagA may act to inhibit gastric epithelial-programmed cell death.

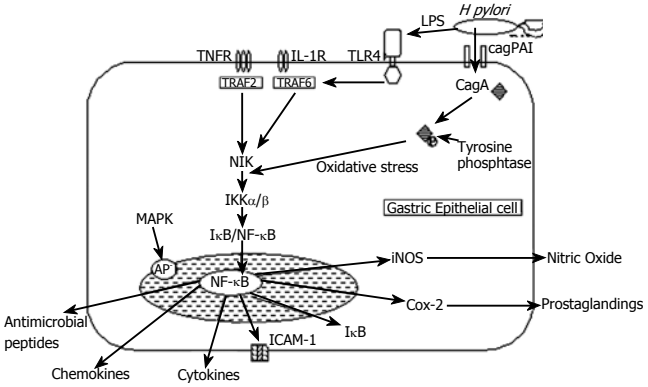


Figure 2 *H pylori* induced gastric epithelial inflammation.

***vac*A gene:** The *vac*A gene encodes the expression of a vacuolating cytotoxin VacA, which induces vacuole formation in eukaryotic cells and stimulates epithelial-cell apoptosis[31,32]. The toxin inserts itself into the epithelial-cell membrane forming a voltage dependent channel through which bicarbonate and organic anions can be released. Unlike the *cag*-PAI, all *H pylori* strains possess the *vac*A gene, although only approximately 50% of strains express the VacA protein. Differences in expression are due to gene sequence variation[33]. Humans infected with VacA expressing *H pylori* demonstrate a greater degree of gastritis than non-expressing strains. *H pylori* infection is invariably associated with elevated gastric epithelial cell proliferation, thought to be a consequence of the epithelial damage.

***bab*A gene:** The *bab*A gene encodes an outer-membrane protein BabA, which binds to fucosylated Lewis B blood group antigen on gastric cells[11,34]. BabA expressing strains adhere more tightly to gastric epithelial cells, and there is significant evidence accumulating that BabA expression may influence disease severity[11]. *H pylori* strains that possess *bab*A, *vac*A and *cag*A carry the highest risk of gastric cancer[11].

***ice*A gene:** A further putative virulence factor has described-*ice*A (induced by contact with epithelium) comprises two main variants *ice*A*1* and *ice*A*2*[35]. However, the function of *ice*A*2* is currently undefined[36]. Significant homology has been found between *ice*A*1* gene and *nla*III a type II restriction endonuclease of *Neisseria lactamica*. Expression of *ice*A*1* is up-regulated by contact of *H pylori* with human gastric epithelial cells and in some populations it is associated with peptic ulcer disease.

Host genetic factors and gastric cancer

It is now well recognised that the development of gastric

cancer and the precursory changes of gastric atrophy and hypochlorhydria are strongly influenced by host genetic factors. The importance of these factors was indicated by the association of Interleukin-1β gene polymorphisms and those affecting its receptor antagonist with an increased risk of developing hypochlorhydria and gastric atrophy in a Caucasian population of gastric cancer relatives, in the presence of *H pylori*[37]. The risk of gastric cancer is also exacerbated by carriage of these polymorphisms. The estimated odds ratio in a logistic regression model for *IL-1B*-511T +/-31C+ and *IL-1RN**2/*2 were 1.6 (95% CI, 1.2-2.2) and 2.9 (95% CI, 1.9-4.4) respectively, in a Caucasian population[38]. The influence of these proinflammatory IL-1β and IL-1 receptor antagonist gene polymorphisms on the development of gastric pre-malignant lesions and carcinoma has subsequently been verified by other investigators[39,40]. IL-1β is relevant to the pathogenesis of *H pylori*-induced inflammation and the subsequent neoplastic process as the expression is up-regulated by the infection, it has proinflammatory actions and it is a powerful inhibitor of acid secretion[41]. Emphasising the multi-factorial nature of *H pylori* related gastric carcinoma, when these polymorphisms are combined with bacterial virulence factors, such as CagA, vacA s1 and vacA m1 positivity, the risk of developing the disease is greatly enhanced[36].

Several other cytokines in addition to IL-1β are involved in the inflammatory response to *H pylori* and are potential host-genetic risk factors. TNF-α is also up-regulated at an early stage after infection and subsequently influences transcription of several mediators[42]. Conversely IL-10 is an anti-inflammatory cytokine that inhibits cell-mediated immune responses. Functional, pro-inflammatory polymorphisms affecting both the genes encoding TNF-α and IL-10 have been associated with an increased risk of non-cardia gastric cancer[43]. The combination of three or four pro-inflammatory cytokine polymorphisms affecting IL-1β (*IL-1B*-511T), IL-1 receptor antagonist (*IL-1RN**2), TNF-α (*TNF-A*-308A) and IL-10 *(IL-10* haplotype ATA) results in a highly significant increased risk of developing non-cardia gastric cancer (OR 27.3 95% CI 7.4-99.8)[43]. More recently a functional pro-inflammatory polymorphism affecting the *IL-8* gene (*IL-8*-251A) has been associated with increased gastric cancer[44-46].

Several other candidate genes have been studied in the attempt to discover associations with the development of gastric cancer. Cytochrome P450 is involved in the metabolism of dietary carcinogens such as *N*-nitrosamines and the *CYP2E1 (c1/c1)* genotype appears to be associated with an increased risk of cardia cancer, particularly in smokers[47]. HLA class II DR-DQ alleles also appear to influence gastric carcinoma development with DRB1*1601 being associated with an increased risk of gastric cancer, particularly in the absence of *H pylori*[48].

THE ROLE OF INFLAMMATION IN GASTRIC CANCER

H pylori-induced inflammation

It is known that *H pylori* is a potent activator of NF-κB in gastric epithelial cells[49-52] (Figure 2). *H pylori* infection causes activation of the NF-κB pathway by a variety of mechanisms which are discussed in more detail below. Activation of NF-κB by *H pylori* induces nuclear translocation, which causes an increase in IL-8 messenger RNA and protein levels[50,53]. This has important implications since other NF-κB responsive genes including pro-inflammatory cytokines have been found in elevated levels in *H pylori* infected gastric mucosa. NF-κB activation is known to regulate cellular growth responses, including apoptosis, and is required for the induction of inflammatory and tissue-repair genes, including macrophage inflammatory protein (MIP)-2, metalloproteinase 3 (MMP3) and vascular endothelial growth factor (VEGF). The NF-κB pathway is also responsible for the generation of several cell adhesion molecules including ICAM-1 whose expression is significantly correlated with an increase in *H pylori* induced gastritis[54].

Mechanism of H pylori-induced activation of NF-κB

Lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria including *H pylori,* is a signalling molecule for the innate immune system and is the main source of inflammation in Gram-negative infections[55]. LPS targets the transmembranous pattern-recognition receptor called toll-like receptor 4 (TLR4), which is expressed on macrophages and monocytes[56]. LPS binding to TLR4 activates signal transduction through MyD88, interleukin-1 receptor associated kinase and TRAF6 to activate the NF-κB and mitogen-activated protein kinase pathways[57-59], which leads to the synthesis and release of inflammatory cytokines such as IL-1, IL-8 and TNF-α, various chemokines GROα, IP10, MIG and MIP -1α & β[60], iNOS[61,62] and antimicrobial peptides providing a critical link to the adaptive immune system[63,64].

Induction of inflammation is an important component in the defence against *H pylori*. The presence of *H pylori* leads to the release of mutagenic substances such as metabolites of inducible nitric oxide synthase (iNOS), which is known to promote oncogenesis[65]. Nitric oxide generated by iNOS is converted to reactive nitrogen species, which can exert oncogenic effects including direct DNA and protein damage, inhibition of apoptosis, mutation of DNA and cellular repair functions such as p53 and also promotion of angiogenesis[66]. *H pylori* infection is also known to induce the expression of pro-inflammatory Cyclooxygenase enzyme (COX-2)[67-71]. COX-2 expression is normally undetectable in most normal tissues, but is induced rapidly during an inflammatory reaction[72,73]. Cox-2 activity is induced by a variety of mediators including inflammatory cytokines such as TNF-α, interferon-γ and IL-1[74]. COX-2 facilitates tumour growth by inhibiting apoptosis, maintaining cell proliferation and stimulating angiogenesis within cancer cells[75-77].

Production of reactive oxygen species

A positive association between ROS production and *H pylori* infective load has been established[78]. It has also been suggested that the source of ROS production was most

probably host neutrophils, which had been activated by the presence of *H pylori*. It has also been shown that ROS production is enhanced by infection with *cag*A-positive *H pylori* strains[79].

MOLECULAR MECHANISMS OF GASTRIC CARCINOGENESIS

The bacterial, environmental and host genetic factors discussed above influence the development of gastric carcinoma. In the following section, we discuss the molecular mechanisms underlying the disease. These include abnormalities of oncogenes, tumour suppressor genes, cell adhesion molecules and cell cycle regulators. Additionally genetic instability and alterations in growth factors and cytokines contribute to the complex pathways involved in gastric carcinogenesis. Differences exist in the pathways leading to diffuse- and intestinal-type gastric carcinoma, and these are summarised in Figures 3 and 4.

Oncogenes

Many proto-oncogenes are activated in gastric carcinoma, with variations between the differing histological subtypes. The *c-met* gene, encoding a receptor for hepatocyte growth factor/scatter factor is amplified in 19% of intestinal-type and 39% of diffuse-type gastric cancers[80]. The majority of gastric carcinomas express two different c-met transcripts, of 7.0 kb and 6.0 kb. Expression of the 6.0kb transcript correlates well with prognostic factors such as tumour staging, depth of tumour invasion and lymph node metastasis[81]. The K-sam (KATO-III cell-derived stomach cancer amplified) oncogene is also frequently activated in gastric carcinomas, and has at least four transcriptional variants[82]. One of these, Type II, encodes a receptor for keratinocyte growth factor. K-sam is preferentially amplified in 33% of advanced diffuse or scirrhous -type gastric carcinomas but not in intestinal-type cancers[83]. Over-expression of this gene in gastric carcinoma is associated with a poorer prognosis.

Another proto-oncogene, *c-erb*B2, is preferentially amplified in 20% of intestinal-type gastric cancers but this is not a feature of the diffuse-type[84]. Over-expression of this gene is also correlated with poorer prognosis and liver metastases[85,86]. Mutations of K-ras are seen in intestinal-type gastric adenocarcinomas and the precursor lesions intestinal metaplasia and adenomas[87-89]. The incidence of this mutation is low and it is not a feature of diffuse-type carcinomas.

Tumour suppressor genes

The tumour suppressor gene p53 is frequently inactivated in gastric carcinoma by loss of heterozygosity (LOH), missense mutations and frame shift deletions. This occurs in over 60% of gastric cancers, regardless of the histological subtype, and is frequently observed in precursor lesions such as intestinal metaplasia, dysplasia and adenomas[88,90-94]. Mutations commonly occur at A:T sites in intestinal-type carcinomas, with GC-AT transitions being common in diffuse-type carcinomas[91]. These GC-AT transitions can be caused by carcinogenic

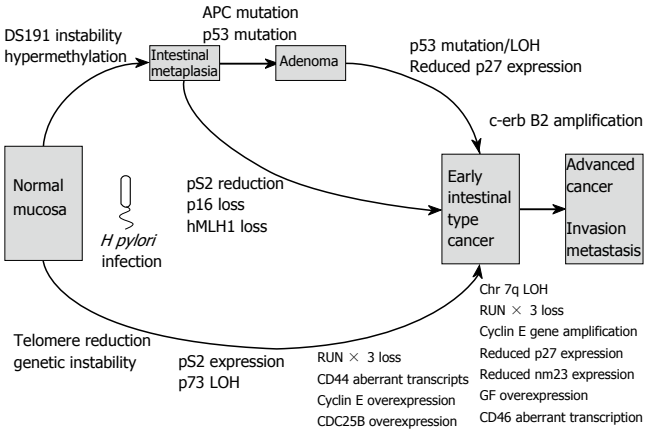


Figure 3 Intestinal type gastric cancer: Genetic and epigenetic alterations during carcinogenesis.

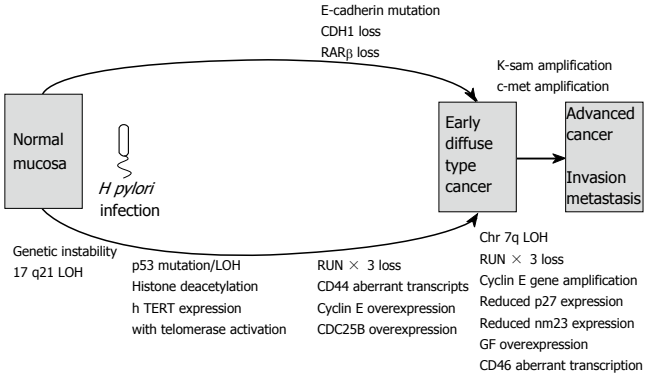


Figure 4 Diffuse type gastric cancer: Genetic and epigenetic alterations during carcinogenesis.

N-nitrosamines that are found in several foodstuffs and can be produced from dietary amines and nitrates in the acidic gastric environment[95,96]. Mutations in the codon 72 of exon 4 of the p53 gene have recently been associated with an increased risk of distal gastric cancer[97]. LOH of p73, a tumour suppressor gene related to p53 is detected in 38% of gastric cancers, and alterations of this gene are predominant features of foveolar-type gastric cancers with pS2 expression[98]. pS2 is a gastric-specific trefoil factor normally expressed in gastric foveolar epithelial cells. Inactivation of the pS2 gene results in dysplasia, adenoma and adenocarcinomas in mice[99,100]. Reduction or loss of the pS2 gene by DNA methylation in the promoter region occurs in intestinal metaplasia and gastric adenomas, suggesting this process may be important at an early stage in intestinal-type gastric carcinoma development[101].

Mutations of the tumour suppressor gene APC, involved in familial polyposis coli, are also observed in intestinal-type gastric carcinoma[102]. Although APC gene missense mutations are common in the intestinal subtype, occurring in over 50% of cases, they are not involved in diffuse-type cancers. Somatic mutations of the APC gene are observed in 20%-40% of gastric adenomas and 6% of intestinal metaplasias[103,104]. The expression of β-catenin, which acts as an oncogene, is enhanced by APC inactivation.

A further tumour suppressor is nuclear retinoic acid receptor β (RARβ). Hypermethylation of this gene with reduced expression is observed in 64% of intestinal gastric cancers but this is not observed in the diffuse subtype[105]. Additional tumour suppressor gene alterations include those affecting distinct chromosomal loci. LOH at 1q and 7q are frequently associated with intestinal-type cancers while 1p is commonly affected in advanced diffuse cancers[88]. LOH of the bcl-2 gene is also frequently observed in intestinal-type cancers[106].

The *RUNX* gene family is composed of three members, *RUNX1/AML1*, *RUNX2* and *RUNX3*[107]. It also encodes the DNA-binding α subunits of the Runt domain transcription factor polyomavirus enhancer-binding protein 2 (PEBP2)/core-binding factor (CBF), which is a heterodimeric transcription factor. Of the RUNX family, *RUNX3* is involved in gastric carcinogenesis, being necessary for the suppression of cell proliferation in the gastric epithelium. The gastric epithelium of *RUNX3* knockout mice exhibits hyperplasia, reduced rate of apoptosis and reduced sensitivity to TGFβ1, suggesting the tumour suppressor activity of *RUNX3* operates downstream of the TGFβ signalling pathways. In humans, loss of *RUNX3* by hypermethylation of the promoter CpG island is observed in several different cancers, including 64% of gastric carcinomas. *RUNX3* methylation is also a feature of 8% of chronic gastritis, 28% of intestinal metaplasia and 27% of gastric adenomas[108]. This suggests *RUNX3* is a target for epigenetic gene silencing in gastric carcinogenesis[109,110].

Other genes that appear to be affected in gastric carcinogenesis include the *FHIT* gene and loss of heterozygosity at the DCC locus, which is a feature of intestinal-type cancers[88,111]. Promoter hypomethylation of a novel cancer/testis antigen gene CAGE has recently been described in 35% of chronic gastritis and 78% of gastric cancer[112]. Histone H4 is progressively deacetylated during the development of gastric cancer, and this is a common event in both intestinal-type and diffuse type cancers[113].

Cell-adhesion molecules and metastasis-related genes

Cell adhesion molecules may act as tumour suppressors, with mutations in the E-cadherin gene occurring preferentially in 50% of diffuse type gastric carcinoma[114]. This homophilic cell adhesion molecule belongs to a family of cell-cell adhesion molecules with an important role in intercellular adhesion by establishing cell polarity, maintaining tissue morphology and cellular differentiation in normal cells[115,116]. E-cadherin binds to the actin cytoskeleton via a series of catenin proteins[117]. Therefore changes in E-cadherin expression have a direct effect on cell adhesion and therefore plays an important step in cancer development. E-cadherin mutations affecting exons 8 or 9 induce the scattered morphology, decreased cellular adhesion and increased cellular motility of diffuse gastric cancers[118]. Mutations in β-*catenin* and γ-*catenin* have also been observed in gastric cancer cell lines, and together with E-cadherin mutations appear to be involved in the development and progression of diffuse and schirrhous-type cancers[119-121].

Abnormal CD44 transcripts are frequently associated with gastric carcinomas and metastatic deposits, with the pattern of these abnormal transcripts varying between the intestinal and diffuse subtypes[122]. All gastric cancer cell lines and tissues exhibit abnormal CD44 transcripts containing the intron 9 sequence[123]. This feature is also observed in 60% of gastric intestinal metaplasias but is absent from normal mucosa[124]. Osteopontin (OPN), a protein ligand of CD44, is overexpressed in 73% of gastric carcinomas and when co-expressed with CD44v9 correlates lymphatic invasion and metastasis[125,126]. Reduced expression of nm23, involved in c-myc transcriptional activation, and galectin-3, a galactoside-binding protein, are also implicated in metastatic gastric carcinoma[127,128].

Cell-cycle regulators

The cell-cycle regulator, cyclin E, is amplified in 15%-20% of gastric carcinomas that are associated with its overexpression. Gene amplification or overexpression of cyclin E are associated with aggressiveness and lymph node metastasis[129]. The expression of the CDK inhibitor p27 that binds to a wide variety of cyclin/CDK complexes and inhibits kinase activity is frequently reduced in advanced gastric carcinoma while being preserved the majority of gastric adenomas and early cancers[130]. Reduced p27 expression correlates with tumour invasion and nodal metastasis. This reduction in p27 occurs at a post-translational level, and results not from genetic abnormalities but rather from ubiquitin-mediated proteosomal degradation[131]. A family of E2F transcription factors is an important target of cyclin/CDKs at the G1/s transition. Overexpression of E2F is observed in 40% of primary gastric cancers, and this tends to be co-expressed with cyclin E[132]. Gene amplification and abnormal expression of the E2F gene may permit the development of gastric cancer.

Micosatellite and chromosomal instability

Microsatellite instability (MSI) is a hallmark of the DNA mismatch repair deficiency that is one of the pathways of gastric carcinogenesis. Microsatellites are short DNA sequence repeats that are scattered throughout the human genome and occur in nearly every case of gastric cancer associated with germline mutations of the mismatch repair (MMR) genes *hMSH2, hMLH1, hPMS1*, *hPMS2*, and *MSH6/GTBP*[133,134]. Errors that occur in DNA mismatch repair mechanisms in tumour cells can cause expansion and contraction of these repeats. MSI due to epigenetic inactivation of *hMLH1* is found in 15%-39% of sporadic intestinal-type cancer, 70% of which are associated with loss of hMLH1 by hypermethylation of the promoter[135,136]. Such intestinal type cancers with MSI often occur in older patients and arise in the antrum. They are associated with lymphocyte infiltration, multiple tumours and a potentially favourable prognosis. Meanwhile MSI of the D1S191 locus is found in 26% of intestinal metaplasia and 46% of intestinal type gastric cancer. An identical pattern of this MSI of D1S191 is observed in adjacent intestinal metaplasia and intestinal type cancer

that suggests the sequential development from the former to the latter[137]. Diffuse type cancers with MSI are more commonly observed in younger patients and have no germline mutations of *hMLH1* and *hMSH2*, with no alteration in BAT-RII[138]. However, these cancers are frequently associated with LOH on chromosome 17q21 including the BRCA1 gene.

Human telomerase reverse transcriptase (hTERT) is an important determinant of telomerase activity, the enzyme that catalyses the telomere DNA synthesis. The majority of intestinal carcinomas have shortened telomere length, high levels of telomerase activity and a significant expression of hTERT[139]. Over 50% of intestinal metaplasias express low levels of telomerase activity, equivalent to 10% of the activity in gastric carcinoma[140]. hTERT is unregulated at an early stage in gastric carcinogenesis and H pylori may act as a trigger factor for hyperplasia in hTERT positive "stem cells" in intestinal metaplasia[139].

Growth factors and cytokines

Gastric cancer cells express a wide array of growth factors and cytokines that act via autocrine, paracrine and juxtacrine mechanisms. Again the expression of these mediators varies depending on the histological subtype. These interactions are summarised in Figure 5. The EGF family, which includes EGF, TGFα, IGF II and bFGF, are commonly overexpressed in intestinal-type carcinoma. Meanwhile TGFβ, IGF II and bFGF are predominantly overexpressed in the diffuse subtype[101]. Co-expression of EGF/TGFα, EGFR and cripto correlates well with the biological malignancy, as these factors induce metalloproteinases[141,142]. Overexpression of cripto is frequently associated with intestinal metaplasia and gastric adenoma[143]. Gastric cancer cells express neutrophilin-1 (NRP-1), a co-receptor for VEGF receptor 2 endothelial cells[144]. EGF induces both NRP-1 and VEGF expression, suggesting that regulation of NRP-1 expression in gastric cancer is intimately associated with the EGF/EGFR system.

Interleukin-1α is produced by inflammatory cells and also gastric cancer cells. It acts as an autocrine growth factor for gastric carcinoma cells and is important in EGF and EGF receptor expression[145]. The interplay between IL-1α and the EGF/receptor system acts to stimulate gastric cancer growth. IL-6 also acts in an autocrine fashion to stimulate gastric cancer cells. Il-1α and IL-6 both stimulate the expression of each other by tumour cells{167}. IL-8, a member of the CXC family of chemokines has numerous roles in gastric carcinogenesis with over 80% of gastric tumours expressing both this cytokine and its receptor[146,147]. IL-8 enhances expression of EGF receptor, type IV collagenase (metalloproteinase (MMP)-9), VEGF and IL-8 mRNA itself by gastric cancer cells, while reducing E-cadherin mRNA expression.

The negative growth factor TGFβ is frequently overexpressed in gastric carcinoma, particularly diffuse type carcinomas with diffusely productive fibrosis[148]. Angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and IL-8 are produced by tumour cells and result in neovascularisation within gastric carcinoma tissue. VEGF promotes angiogenesis and progression of gastric carcinomas, particularly those of the intestinal subtype, while bFGF is has a stronger association for diffuse gastric carcinoma[149,150]. HGF/SF (hepatocyte growth factor/scatter factor) is produced by stimulated stromal cells such as fibroblasts, and functions in a paracrine manner as a morphogen or motogen[101].

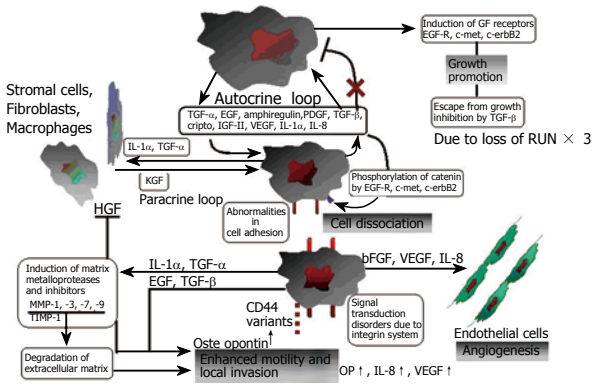


Figure 5 Cancer-stromal interaction in gastric cancer through growth factors and cytokines.

It can be seen that several of the molecular mechanisms are distinct for intestinal- and diffuse-type gastric carcinoma development, while some are common to both. Regarding intestinal-type carcinogenesis, there are three possible routes leading to carcinoma development. Firstly, progression through the pre-cancerous lesions of intestinal metaplasia to adenoma and finally carcinoma. Secondly, intestinal metaplasia may proceed directly to carcinoma. The third route involves the development of de novo gastric carcinoma with no preceding stage.

Summary of Molecular Mechanisms involved in gastric carcinogenesis

The first two pathways are summarised in Figure 3 and Figure 4. Genetic instability and hyperplasia of hTERT positive stem cells precede replication error at the DS191 locus, DNA hypermethylation at the D17S5 locus, pS2 loss, RARβ loss, RUNX3 loss, CD44 abnormal transcripts and p53 mutation, all of which accumulate in 30% of incomplete intestinal metaplasia. All of these epigenetic and genetic changes are common events in intestinal-type cancers.

An adenoma to carcinoma sequence is observed in around 20% of gastric adenomas with APC mutations. Molecular events associated with this sequence are loss of heterozygosity and mutation of p53, reduced p27 expression, loss of RUNX3, over-expression of cyclin E and abnormal c-met transcription. The resulting advanced intestinal-type gastric carcinomas frequently exhibit DCC loss, APC mutations, 1qLOH, loss of p27, reduced TGFβ receptor expression, reduced nm23 and c-erbB2 gene amplification. The "de novo" pathway of gastric carcinoma development involves LOH and abnormal expression of p73 exhibited in the development of foveolar-type gastric cancers. Meanwhile, diffuse-type gastric carcinogenesis

involves LOH at chromosome 17p, MOH or mutation of p53, RUNX3 loss and mutation or loss of E-cadherin. Several of the above molecular events may be present in mixed gastric cancers that have both intestinal and diffuse components.

CONCLUSION

It is evident that gastric carcinoma results from a complex interaction between bacterial, environmental, host-genetic and molecular mechanisms. The importance of *H pylori* and the mechanisms by which the host recognises and responds to the bacterium are crucial in determining the resulting phenotype. Several common events are shared between the differing histological subtypes while distinct differences also highlight the fascinating divergence in histogenesis. The aetiology of these differences remains to be elucidated. Better understanding of these events will no doubt contribute significantly to our fight against this killer cancer. More importantly, the improved understanding of the pathogenesis of gastric cancer will serve as a paradigm for understanding other human cancers.

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REVIEW

Helicobacter pylori infection causes gastric cancer? A review of the epidemiological, meta-analytic, and experimental evidence

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Abstract

Since the discovery of *Campylobacter*-like organisms *Helicobacter pylori* (*H pylori*) more than two decades ago the possibility of a relationship with gastric cancer has been postulated, tested and supposedly proven. There have been numerous human studies of various designs from many countries around the world. Several meta-analyses have been published and more recently a small number of experimental animal studies were reported looking at the association between *H pylori* infection and gastric cancer. Over the years, the human epidemiological studies have produced conflicting results; the meta-analyses have as one would expect produced similar pooled estimates; while the early experimental animal studies require replication. The exact mechanisms by which *H pylori* might cause gastric cancer are still under investigation and remain to be elucidated.

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Key words: *Helicobacter pylori*; Gastric cancer; Meta-analysis; Systematic review

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INTRODUCTION

"They (Helicobacter pylori) may have a part to play in other poorly understood gastritis associated diseases (ie, peptic ulcer and gastric cancer)." [1]

The suggestion of a link between *Helicobacter pylori (H pylori)* infection and gastric cancer was first proposed by co-discoverer Marshall in 1983[1]. It is of interest that at that time the majority of physicians and scientists around the world did not even believe that there was a link between *H pylori* and gastritis[2,3]. Just over a decade later in 1994, the International Agency for Research on Cancer (IARC) classified *H pylori* as a definite class 1 carcinogen[4], despite some conflicting results[5,6]. No experimental studies had been conducted at that time and the epidemiological studies that had been done produced mixed results. Almost all of the studies did not take into consideration other important potential confounders or sources of interaction (e.g., salt intake, diet, alcohol consumption, and smoking). A number of additional studies have also failed to detect an association even in high risk populations[7,8], which has led some to question whether there is a causal link[9]. However, these epidemiological observations appear to be directly supported by early data from animal models on cancer development following *H pylori* infection[10].

This review addresses some of the controversies in the expanding literature supporting a positive association for *H pylori* infection leading to gastric cancer. This review will not address the relationship between *H pylori* and mucosa-associated lymphoid tissue (MALT) lymphoma. An excellent review on this topic has recently been published[11]. Specifically, this review aims to assess the epidemiological evidence based on Bradford Hill's criteria of causality[12], experimental data using Henle-Koch's[13] postulates (Evan's postulates will not be reviewed)[14] and summarize the meta-analytic evidence for the association between *H pylori* and gastric cancer.

MATERIALS AND METHODS

The search strategy involved the major computer databases, including Medline, PubMed, EMBASE and Current Contents (January 1983 to February 2006). The search methodology involved using combinations of the following keywords: *H pylori*, *Camplyobacter pylori*, *Campylobacter pylordis*, gastric cancer, gastric carcinoma, stomach cancer, meta-analysis, systematic review, gastric adenocarcinoma, experimental, animal studies, systematic review. Additional manual searches were made using the reference lists from the selected articles to retrieve other papers relevant to the topic. No language restriction was placed on any of the literature searches.

RESULTS

The IARC-WHO report

In 1994, The International Agency for Research on Cancer (IARC) published a report based on a meeting of experts between 7-14 June of that year in Lyon, France[4]. In terms of a link between *H pylori* and gastric cancer, the conclusions reached in this report stated that there was sufficient evidence for carcinogenicity among humans and inadequate evidence for carcinogenicity in animals, with an overall evaluation classifying *H pylori* as a group 1 definite carcinogen. Sufficient evidence relating carcinogenicity in humans as stated in the report is: "Sufficient evidence of carcinogenicity: The Working Group considers that a causal relationship has been established between exposure to the agent, mixture or exposure circumstance and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence." Inadequate evidence relating carcinogenicity in animals as stated in the report is: "Inadequate evidence of carcinogenicity: The studies cannot be interpreted as showing either the presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations, or no data on cancer in experimental animals are available."

In the overall evaluation a Group 1 carcinogen as stated in the report is: "Group 1 - The agent (mixture) is carcinogenic to humans: The exposure circumstance entails exposures that are carcinogenic to humans: This category is used when there is sufficient evidence of carcinogenicity in humans. Exceptionally, an agent (mixture) may be placed in this category when evidence in humans is less than sufficient but there is sufficient evidence of carcinogenicity in experimental animals and strong evidence in exposed humans that the agent (mixture) acts through a relevant mechanism of carcinogenicity." The findings of the IRAC were four years before the published experimental animal data or meta-analysis of the human epidemiological studies[10,15]. So why was *H pylori* given such a classification? Would another classification have been more appropriate and if so, which one?

The overall IARC evaluation options (Group 1, Group 2A, Group 2B, Group 3, Group 4) appear to assume that there are usually experimental animal data but may not be sufficient human data to support that an agent is carcinogenic to humans. However, in the case of *H pylori* and gastric cancer the opposite is actually true. There were human studies, but at that point in time (1994) there were no actual animal or experimental data. From a cause and effect point of view, one would think that animal data would be essential to link an agent with causing carcinogenic effects, but this does not occur with *H pylori*. Of all the agents assessed in the IARC monograph, only *H pylori* with inadequate animal evidence but 'sufficient human evidence' was classified as a class 1 definite carcinogen (Table 1). All the other agents except for *H pylori* and *Opisthorchis felineus* had at least a limited amount of animal data on which an overall evaluation regarding their carcinogenicity to humans was based. Therefore, based on the data available in 1994 regarding *H pylori* and gastric cancer, it might appear that the IARC prematurely classified *H pylori* as a definite class 1 carcinogen.

Table 1 IARC evaluation of schistosomes, liver flukes and *H pylori*

Agent (infection with)	Degree of evidence of carcinogenicity		Overall evaluation
	Human	Animal	
Schistosoma haematobium	Sufficient	Limited	Definite carcinogen
Schistosoma japonicum	Limited	Limited	Possible carcinogen
Schistosoma mansoni	Inadequate	Limited	Not classifiable
Opisthorchis viverrini	Sufficient	Limited	Definite carcinogen
Opisthorchis felineus	Inadequate	Inadequate[1]	Not classifiable
Clonorchis sinensis	Limited	Limited	Probable carcinogen[2]
Helicobacter pylori	Sufficient	Inadequate[1]	Definite carcinogen

[1] No data available. [2] Other relevant data taken into account in making overall evaluation.

This is because the available epidemiological evidence was insufficient in quality (confounders not adequately assessed) and quantity to establish causal link and there were no experimental animal studies at that time.

Epidemiological data

In 1965, Bradford Hill (1897-1991) published his seminal paper titled "The Environment and Disease: Association or Causation?"[12] This paper was initially written to assess causal links between sickness and injury related to work. Bradford Hill's criteria will be used to assess the possibility of a causal association between *H pylori* and gastric cancer[16].

Consistency: The association is consistent when results are replicated in studies in different settings using different methods. The human epidemiological studies have produced mixed results with approximately 50% finding a positive association between *H pylori* infection and gastric cancer and 50% reporting a negative relationship[15,17,18]. This variation also exists within countries and between countries[15,17,18]. The majority of studies reported were case-control in design with a smaller number of cohort (usually nested case-control) studies which also provided mixed results[15,17,18].

Strength: This is defined by the size of the risk as measured by appropriate statistical tests. The strength of the relationship between *H pylori* and gastric cancer varies quite considerably. Negative studies have produced results as low as (OR: 0.54, 95% CI: 0.24-1.19)[6], and positive studies as high as (OR: 13.3, 95% CI: 5.30-35.60)[19]. The average strength of the relationship as determined by meta-analysis produces an effect size (odds ratio) of approximately 2.00[15,17,18].

Specificity: This is established when a single putative cause produces a specific effect. *H pylori* is predominantly found in the stomach and is associated with gastritis, peptic ulcer disease, and gastric cancer; however, it is linked with

Table 2 Globocan 2002 incidence, mortality, and survival data for gastric cancer

	Incidence			Mortality			Prevalence	
Males	***n***	**CR**	**ASR**	**Deaths**	**CR**	**ASR**	**1-year**	**5-year**
World	603 419	19.3	22	446 052	14.3	16.3	302 067	951 403
Developed	195 782	33.7	22.3	128 721	22.2	14.5	117 377	409 777
Under-developed	405 211	15.9	21.5	315 603	12.4	17	184 690	541 626
Females								
World	330 518	10.7	10.4	254 297	8.3	7.9	166 436	522 156
Developed	115 372	18.8	10	83 515	13.6	6.9	66 319	227 316
Under-developed	214 024	8.7	10.4	169 971	6.9	8.3	100 117	294 840

CR: Crude rate; ASR: Age standardized rate (World Standard).

numerous extra-gastric conditions including foetal intra-uterine growth restrictions[20], glaucoma[21], Raynaud's phenomenon[22], gallstones[23], primary headache[24], diabetes[25], ischemic heart disease[26], Sjögren's syndrome[27], Schönlein-Henoch purpura[28], autoimmune thyroiditis[29], chronic bronchitis[30], Parkinson's disease[31], non-arterial anterior optic ischemic neuropathy[32], chronic idiopathic urticaria[33], rosacea[34], alopecia areata[35], sideropenic anemia[36], growth retardation[37,38], late menarche[39], hepatic encephalopathy[40], and stroke[41]. Thus, currently *H pylori* is not associated with a single specific condition or disease within or outside the human stomach[42-46]. It must be remembered that none of these conditions are causally linked with *H pylori*.

Dose-response relationship: An increasing level of exposure (in amount and/or time) increases the risk. It is well known that infection with *H pylori* involves an inflammatory process occurring over many years[47-51], thus, the risk of gastric cancer may correlate with the duration of infection with *H pylori*. Therefore, those individuals who are infected during childhood have a longer time to develop mutations compared to those who acquire infection during adulthood. It has been suggested that a child's stomach might be physiologically different from an adult's stomach, because children have less acute inflammation and an increased number of lymphoid follicles than adults[52]. However, the amount of organisms living in a human's stomach has never been correlated with pathology.

Temporal relationship: Exposure always precedes the outcome. This is the only absolutely essential criterion. Current epidemiological evidence suggests that infection is usually acquired during childhood and is attributed to a cohort effect[53-57]. A recent study showed that age of acquisition after one year of age is not the most important factor related to the differences in incident rates of gastric cancer in adulthood[58]. However, studies have also shown that re-infection with *H pylori* is also possible, even though it may occur infrequently[59, 60].

Biological plausibility: The association agrees with currently accepted understanding of pathobiological processes. This criterion should be applied with caution. The prospect that cancer might be spread via an infectious mechanism has been considered for centuries[61,62]. Moreover, over the last decade there has been an exponential increase in the scientific knowledge related to infectious agents associated with cancer[63-65]. There are a large number of infectious agents associated with cancers including Epstein-Barr virus (EBV), human papillomaviruses (HPVs), hepatitis B or C virus, human herpesvirus type 8 (HHV-8), *Salmonella typhi/paratyphi*, *Schistosoma hematobium*, polyoma viruses[66-72]. Over the last decade there has certainly been an enormous increase in the amount of data on association between *H pylori* and gastric cancer, with numerous studies and hypothetical mechanisms explored[73-77]. However, while it is definitely plausible that *H pylori* could cause gastric cancer, at present the exact mechanism remains unknown.

Coherence: The association should be compatible with existing theory and knowledge. Epidemiologically, at times, there are clear signs of a relationship between *H pylori* and gastric cancer, for example, studies that reported a high prevalence of *H pylori* among a population with a high rate of gastric cancer (e.g., Japan)[9,78,79]. Conversely, there are times when a high prevalence of *H pylori* correlated with the low rate of gastric cancer (e.g., Africa)[9,79,80]. However, one African study reported that there was no difference in prevalence of *H pylori* infection among gastric cancer cases and non-ulcer dyspepsia controls[81]. The fact that only approximately 1% of those infected with *H pylori* actually go on to develop gastric cancer suggests a lack of coherence. Of course there are exceptions; in Changle, China, up to 20% of *H pylori* infected individuals developed gastric cancer. Should not all those infected with *H pylori* eventually develop gastric cancer? Indeed, if 50% of the world is infected then this equates to 30 million cases of gastric cancer, which is a gigantic number of potentially preventable malignancies. The latest epidemiological data from GLOBOCAN 2002 (data reported 2-5 years earlier) reported that there were 933 937 incident cases and 700 349 deaths due to gastric cancer in that year (Table 2)[79]. Histopathologically, there is a paradox where *H pylori* organisms are not found in the stomachs of gastric cancer cases, which may explain the lower antibody response among these patients[82-84]. These are arguments against coherence for the causal relationship between *H pylori* and gastric cancer.

Experiment: The condition can be altered by an appropriate experimental regimen. This is possibly the most important support for a causal relationship. Thus,

the strongest proof for a link between *H pylori* and gastric cancer would be established only if controlled trials demonstrate that elimination or prevention of *H pylori* infection prevents malignancy. An animal study has documented that among Mongolian gerbils treated with *N*-methyl-*N*-nitrosourea (MNU) and infected with *H pylori* where 2 of the 6 groups underwent eradication of *H pylori* infection at 21 weeks, after 50 weeks they showed a decrease in the number of gastric cancers developed[85].

Recently, a human prospective randomized, placebo-controlled prevention trial compared patients who received eradication therapy for *H pylori* (n = 817) and those taking placebo (n = 813) and at follow-up (7.5 years) determined the incidence of gastric cancer between the groups[86]. However, there was no significant difference in gastric cancer incidence between those who received eradication therapy and placebo (P = 0.33). This study was criticised for being stopped early, as it was believed that if the study had continued for a few more years it might have yielded more conclusive results[87]. Kamada *et al*[88] conducted a prospective non-randomized follow-up study of 1787 patients who underwent *H pylori* eradication therapy and were followed up after 9 years. Gastric cancer occurred in 20 patients (1.1%), while early gastric cancer consisted of 5.7% (6/105) after endoscopic resection. This study concluded that even after successful eradication therapy for *H pylori* endoscopic assessment should be undertaken for occult early gastric cancer or severe mucosal atrophy. Moreover, studies trying to determine if gastric atrophy is a reversible process have produced conflicting results[89]. Thus, endoscopic screening in high risk populations still remains appropriate[90,91]. It should be remembered that epidemiological evidence by itself is insufficient to establish causality, but it does provide strong circumstantial evidence.

Meta-analysis

At present, five meta-analyses have been published assessing the relationship between *H pylori* and gastric cancer[15,17,18,92,93], and one that focuses specifically on the CagA cytotoxin, will be discussed separately[94]. An additional earlier combined-analysis[95] which contained only three early (all published in 1991) prospective studies produced a pooled odds ratio of 3.80 (95% CI: 2.30-6.20). This paper will not be discussed in this review due to the lack of information regarding this particular combined-analysis. These meta-analyses vary in several different ways: (1) the number of studies included in the analysis; (2) the types of studies included; (3) whether the studies were peer-reviewed or in abstract form; (4) published language of the studies; (5) databases used; (6) the search terms used; (7) method of *H pylori* diagnosis; (8) statistical analyses used; (9) statistical heterogeneity; (10) if quality assessment was undertaken; (11) determination of publication bias; (12) how to deal with duplicate studies; and (13) the number of reviewers involved in data extraction. Of the five meta-analyses that assessed the association between *H pylori* and gastric cancer the number of studies included in each of the meta-analyses varies mostly due to study design. The number of studies included ranged between 11-44. Moreover, the number of studies used in the statistical analysis is exactly the same as the number of studies included except for one meta-analysis where the analysis was undertaken based on 10 out of the 44 studies (Table 3)[18]. Three of the meta-analyses combined both case-control and cohort studies[15,17,18], whereas, two meta-analyses contained only case-control studies[15, 17].

Two meta-analyses placed no language restrictions on the publications included[17,18], one incorporated only English language publications[15] while another only used those published in Chinese[92]. The types and number of databases used differed with the majority using only one database[15,18,92,93], and one meta-analysis using five databases[17]. Only three meta-analyses incorporated manual searches of reference lists[15,17,18], one searched conference proceedings[15], and two contacted investigators[17,93]. The meta-analyses also used assorted search terms varying in number between 3-5. Three meta-analyses only used studies that determined *H pylori* status by serology[15,18,93] while one used a combination of serology and histology[17] and one did not state the method of *H pylori* determination[92]. Statistical methods also differed with four of the meta-analyses using a combination of a fixed and random-effects procedure. The pooled estimates from these five meta-analyses ranged between 1.92-2.56 (mean 2.28) with confidence intervals ranging between 1.35-3.55. Quality assessment was only undertaken in two of the meta-analyses[15,17], with only one using quality scores[17]. In addition, publication bias was only determined in one of the meta-analyses[17]. Duplicate studies were removed in two of the meta-analyses[15,17], and not stated in the other meta-analyses. The number of assessors also differed between the meta-analyses ranging between one and three and two studies having an unknown number of assessors.

The additional meta-analysis, focused on the relationship between cagA seropositivity and gastric cancer[94]. Previous studies have reported a higher prevalence of cagA-positive strains among populations with a high incidence of gastric cancer[94]. This meta-analysis included 16 case-control studies, and there were no language restrictions used on the literature search which included the databases of Medline and PubMed that only assessed publications between the years 1990-2003. The study produced a pooled odds ratio of 2.28 with a confidence interval ranging between 1.71-3.05. However, there was significant heterogeneity among the studies included in this analysis. A quality assessment was undertaken but did not involve producing a score for each particular study included in the meta-analysis. The authors did assess publication bias, while duplicate studies were removed and three authors conducted the literature searches. One meta-analysis[17] found that younger subjects were at lower risk (OR 0.77, 95% CI: 0.68-0.89) of developing gastric carcinoma if infected with *H pylori*, which is supported by the epidemiological data. However, another meta-analysis[15] showed that the odds ratios decreased significantly with increasing age (9.29, 3.43-34.04 aged 20-29 years; 1.05, 0.73-1.52 aged $\geqslant$ 70 years). The reason for this difference may be due to the staging of

Table 3 Summary of all meta-analyses looking at the association between *H pylori* and gastric cancer

Study Factors	Huang *et al* 1998[16]	Danesh 1999[19]	Eslick *et al* 1999[18]	Xue *et al* 2001[93]	HCCG 2001[94]	Huang *et al* 2003[95]
Journal	Gastroenterology	Aliment Pharm Ther	Am J Gastroenterol	World J Gastroenterol	Gut	Gastroenterology
Number of studies	19	44	42	11	12	16
Cohort studies	5	10	8	0	0	0
Case-control studies	14	34	34	11	12	16
Peer-reviewed papers	18	37	42	†	12	12
Abstracts	1	7	0	†	0	4
Non-English language studies	English only	No restrictions	No restrictions	Chinese only	†	No restrictions
Searching method(s)	MEDLINE, Conference proceedings (1994-1996), manual searches	MEDLINE, Reference lists, hand searching	MEDLINE, Current contents, CINAHL, CANCER CD, Biological abstracts, manual searches, contact investigators	Chinese Biomedical database (CBM)	MEDLINE, contact investigators	MEDLINE, PubMed, contact investigators
Search terms used	Campylobacter pylori, stomach neoplasms, Helicobacter pylori	Campylobacter pylori, gastric adenocarcinoma, gastric cancer, Stomach cancer, Helicobacter pylori	Campylobacter pyloridis, Campylobacter pylori, gastric carcinoma, gastric cancer, Helicobacter pylori	Helicobacter pylori, gastric carcinoma, gastric cancer, precancerous lesion of stomach	†	Helicobacter pylori, meta-analysis, stomach neoplasms
Time period	1983-1996	1983-1998	1983-March 1999	1995-2001	1985-1999	1990-2003
***H pylori* status**	Serology only	Serology only	Serology & histology	†	Serology	Serology
Statistical analysis	Random-effects M-H or D&L	Inverse-variance-weighted log risk odds	Random-effects D&L	Fixed-effects	Logistic regression	Random-effects D&L
Number of studies in analysis	19	10	42	11	12	16
Pooled odds ratio (OR)	1.92	2.5	2.04	2.56	2.36	2.28
95% confidence interval (CI)	1.32-2.78	1.90-3.40	1.69-2.45	1.85-3.55	1.98-2.81	1.71-3.05
Overall (*P*-value) heterogeneity	0.2	> 0.05	0.001	> 0.05	0.09	< 0.001
Quality assessment	Yes	No	Yes	No	No	Yes
Assessment of publication bias	No	No	Yes	No	†	Yes
Duplicate studies	Removed	†	Removed	†	†	Removed
Number of reviewers	Two	One	Two	†	†	Three

† Not stated. D&L: DerSimonian & Laird [15]. M-H: Mantel-Haenszel [16].

gastric cancer and the difficulty in diagnosing *H pylori* infection in the advanced stages where the normal gastric mucosa has been destroyed.

All these meta-analyses showed that *H pylori* infection is associated with approximately a two-fold increased risk of developing gastric cancer (Figure 1). The strength and consistency of these meta-analyses in terms of a lack of heterogeneity (as only one meta-analysis reported heterogeneity) suggests that these observations are reliable. One of the possible reasons for this heterogeneity might be the variation in study design as both case-control and cohort studies were included.

Animal experimental data

In order to assess a causal relationship experimentally between *H pylori* and the formation of gastric cancer a systematic approach similar to that applied in epidemiological studies is essential. Historically, Henle-Koch postulates (1877 and 1882)[13] have been used to determine causal relationships, however, these postulates were revised in 1976 by Evans (Evans 1976)[14]. This review will only use Henle-Koch postulates to assess this relationship. These were developed in 1877 by Jacob Henle and subsequently revised in 1882 by Robert Koch. The postulates from Last[16] are: The agent must be shown to be present in every case of the disease by isolation in pure culture. As mentioned previously, not all animals or humans infected with *H pylori* go on to develop gastric cancer[10]. At present 50% of the animal studies have used carcinogens in combination with *H pylori* infection[96-98] and the remainder have only used the organism in an attempt to induce gastric cancer[10,99,100].

The agent must not be found in cases of other disease. *H pylori* is associated with other gastrointestinal diseases (chronic gastritis, gastric ulcer, duodenal ulcer, and gastric MALToma) and also a large number of extra-gastric diseases[20-46]. Thus, currently *H pylori* is not associated with a single specific condition or disease within or outside

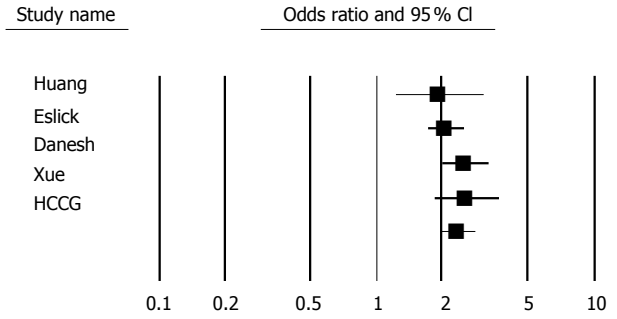


Figure 1 Odds ratios and 95% confidence intervals for individual meta-analyses.

the human stomach. However, it must be highlighted that none of these disease conditions have as yet been definitively/causally linked with *H pylori* infection.

Once isolated, the agent must be capable of reproducing the disease in experimental animals. Marshall's classic back-to-back publications which were published in 1985 in the *Medical Journal of Australia* where the authors aimed to fulfill Koch's postulates[101,102]. These publications described the now famous experiment where Dr Marshall ingested a 10 mL suspension of approximately 10^9 colony forming units of *H pylori* which had been isolated from a patient with gastritis (then referred to as *pyloric Campylobacter*). Over the course of the next ten days, the subject developed symptoms (increased abdominal peristalsis, epigastric fullness, vomiting, headache, soft faeces, and halitosis). A gastroscopy was performed and biopsies taken for both light and electron microscopy, which revealed gastritis and spiral organisms (*pyloric Campylobacter*). These organisms were cultured and freeze-dried. The authors were able to fulfil Koch's postulates in humans for gastritis[101,102]. However, it is not possible to complete Koch's postulates for gastric cancer in humans. It was not until 1998 when the first report by Watanabe *et al*[10] of *H pylori* leading to gastric carcinogenesis that animal models could be assessed in terms of Koch's postulates. It has been suggested that these postulates have been fulfilled for *H pylori* and gastric cancer[103], however, no studies to date have specifically undertaken or successfully proven Koch's postulates using animal models. It appears that these statements are not based on the strict adherence to these postulates of causation, but rather that 'sometimes' *H pylori* is associated with gastric cancer.

The agent must be recovered from the experimental disease produced. It is not always possible to isolate *H pylori* organisms from gastric cancer tissue whether this be from humans or animals[104]. This is due to the histopathological changes that occur which decrease the ability of *H pylori* to adhere to the stomach and thus over time leads to a decreased antibody response[93,105,106].

CONCLUSION

Currently, the epidemiological data are conflicting on the relationship between *H pylori* and gastric cancer. The independent meta-analyses provide an overall consensus that *H pylori* infection is associated with an increased risk (approximately a two-fold increased risk) of developing gastric cancer even though 50% of the studies have produced negative findings. However, there is still a lack of 'cause and effect' evidence concerning this relationship, which requires further animal and epidemiological studies to determine the possible carcinogenic mechanisms by which *H pylori* might cause gastric cancer. One of the major problems in determining a true causal association with *H pylori* and a disease is related to its high world-wide prevalence making associations with many conditions possible. Only strict adherence to Bradford Hill's criteria, Henle-Koch's and/or Evan's postulates ensures that cause-and-effect relationships are correctly identified. Prevention studies in the form of *H pylori* eradication randomised placebo controlled trials among gastric cancer patients are currently being conducted.

DEDICATION

I would like to dedicate this manuscript to Professor Barry Marshall and Emeritus Professor Robin Warren who were awarded the 2005 Nobel Prize for Medicine or Physiology for their discovery of *H pylori*. This finding changed many of our lives and made the practice of clinical gastroenterology more challenging and gastrointestinal research more extraordinary, with advances in numerous areas of medicine (*New Engl J Med* 2005; 353: 2421-2423).

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GASTRIC CANCER

Selective inhibition of cell growth by activin in SNU-16 cells

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Abstract

AIM: To investigate whether activin regulates the cell proliferation of human gastric cancer cell line SNU-16 through the mRNA changes in activin receptors, Smads and p21$^{CIP1/WAF1}$.

METHODS: The human gastric cancer cell lines were cultured, RNAs were purified, and RT-PCRs were carried out with specifically designed primer for each gene. Among them, the two cell lines SNU-5 and SNU-16 were cultured with activin A for 24, 48 and 72 h. The cell proliferation was measured by MTT assay. For SNU-16, changes in ActRIA, ActRIB, ActRIIA, ActRIIB, Smad2, Smad4, Smad7, and p21$^{CIP1/WAF1}$ mRNAs were detected with RT-PCR after the cells were cultured with activin A for 24, 48 and 72 h.

RESULTS: The proliferation of SNU-16 cells was down regulated by activin A whereas other cells showed no change. Basal level of inhibin/activin subunits, activin receptors, Smads, and p21$^{CIP1/WAF1}$ except for activin βB mRNAs was observed to have differential expression patterns in the human gastric cancer cell lines, AGS, KATO III, SNU-1, SNU-5, SNU-16, SNU-484, SNU-601, SNU-638, SNU-668, and SNU-719. Interestingly, significantly higher expressions of ActR IIA and IIB mRNAs were observed in SNU-16 cells when compared to other cells. After activin treatment, ActR IA, IB, and IIA mRNA levels were decreased whereas ActR IIB mRNA level increased in SNU-16 cells. Smad4 mRNA increased for up to 48 h whereas Smad7 mRNA increased sharply at 24 h and returned to the initial level at 48 h in SNU-16 cells. In addition, expression of the p21$^{CIP1/WAF1}$, the mitotic inhibitor, peaked at 72 h after activin treatment in SNU-16 cells.

CONCLUSION: Our results suggest that inhibition of cell growth by activin is regulated by the negative feedback effect of Smad7 on the activin signaling pathway, and is mediated through p21$^{CIP1/WAF1}$ activation in SNU-16 cells.

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Key words: Human gastric cancer cell lines; Activin A; Cell proliferation; Activin receptors; Smads; p21$^{CIP1/WAF1}$

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INTRODUCTION

Inhibins and activins, members of the transforming growth factor-β (TGF-β) superfamily, are polypeptides that were originally isolated from ovarian fluid, based on their effect on pituitary follicle-stimulating hormone (FSH) production. Inhibins are heterodimers that are composed of a common α subunit and one of the two homologous β subunits (βA and βB). Activins are either heterodimers or homodimers of inhibin β subunits (βAβA, βBβB, and βAβB)[1-3].

Activin plays an important role in the proliferation, differentiation, and apoptosis of target cells. The biological activity of activin is mediated by receptor complexes consisting of two different types of receptors, the type I (ActR I) and type II (ActR II) activin serine/threoine kinase receptors[4]. ActR II binds to activin independently of ActR I, but is unable to signal without ActR I. The formation of heteromeric complexes of ActR I and ActR II is required for the mediation of the cellular signal[5]. ActR I and ActR II mRNAs have been widely detected in several human tissues, including prostate cancer, breast cancer, and epithelial ovarian cancer[6-8].

Smad proteins have been known as intracellular signaling mediators of the activin[9, 10]. Smad1, Smad2, Smad3, and Smad5 proteins are phosphorylated by activated specific type I serine/threonine kinase receptors, and thus act in a pathway-restricted fashion. Smad4 protein forms hetero-oligomeric complexes with pathway-restricted Smad proteins, which translocate into the nuclei and activate transcriptional responses. Smad6 and Smad7 function as inhibitors of TGF-β family signaling, including activin[11-13]. Smad7 selectively interferes with different

activin signaling pathways and inhibits erythroid leukemia cells and liver Hep3B cells by blocking intracellular activin signaling[14, 15]. In addition, the mitotic inhibitor $p21^{CIP1/WAF1}$ thas suppresses the growth of malignant cells *in vitro* and *in vivo* can be up-regulated by activin[16-18].

This study was to investigate whether activin can affect cell proliferation in human gastric cancer cell line SNU-16 through the changes in activin receptors, Smads and $p21^{CIP1/WAF1}$ mRNA levels.

MATERIALS AND METHODS

Cell culture and reagents

Human gastric cancer cell lines AGS and KATO III were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). SNU-1, SNU-5, SNU-16, SNU-484, SNU-601, SNU-638, SNU-668, and SNU-719 cells were supplied from the Korean Cell Line Bank. Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS, GIBCO), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 ℃ in a humidified 50 mL/L CO_2 and 95% air. The appropriate amount of recombinant human activin A (activin A, R & D systems, Minneapolis, MN) was added to the culture media.

MTT assay

Cell proliferation was measured with CellTiter 96 Aqueous One Solution (Promega, Madison, WI). Cells were seeded at 5×10^3 cells/well in 6-well plates and incubated with different concentrations of activin A (0, 10, 50, and 100 ng/mL) at 37 ℃ for 24, 48, and 72 h respectively. Cell viability was determined by a colorimetric assay with PMS/MTS (20 μL/well). The absorbance was determined at 492 nm with background subtraction at 650 nm.

Cell number assay

For the cell viability assay, cells were seeded at 5×10^4 cells/mL in 6-well plates. The cells were cultured with 100 ng/mL of activin A for 24, 48, and 72 h respectively. Then cells were washed with phosphate buffered saline (PBS) and resuspended in PBS. Viable cells were counted by the trypan blue exclusion method at each time using a hemocytometer.

RNA extraction and RT-PCR procedures

Total RNA was extracted from cultured cells using the RNAzol B solution kit following the manufacturer's protocol (Tel-test, Friendswood, Texas). First-strand cDNA synthesis was performed using a cDNA synthesis kit (Roche, Mannheim, Germany). cDNA synthesis was performed by reverse transcription in a total volume of 20 μL reaction mixture containing 1 μg RNA, 1× reaction buffer, 1 mmol/L dNTP, 5 μmol/L oligodT, 20 units of RNase inhibitor, and 20 units of AMV reverse transcriptase. The reaction mixture was incubated at 42 ℃ for 1 h, terminated by heating at 95 ℃ for 5 min. Polymerase chain reaction (PCR) was performed with 5 μL (α, βA, βB subunits, and $p21^{CIP1/WAF1}$) or 1 μL (activin receptors, Smads and β-actin) cDNA in a 50 μL reaction mixture of 1× PCR buffer, 0.2 mmol/L of each dNTP, 20 pmoL primer, and 1 unit of Taq DNA polymerase (Roche). Primer sequences are shown in Table 1. The conditions for amplification were as follows: first denaturation at 95 ℃ for 4 min, then denaturation at 95 ℃ for 30 s, annealing at 50 ℃ to 62 ℃ for 30 s, and extension at 72 ℃ for 1 min for 30 cycles (25 cycles for β-actin) and a final extension at 72 ℃ for 7 min. Ten μL of PCR products was separated by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized by image analysis (Gel Doc 1000 gel document system, Bio-Rad, Hercules, CA). The intensities of PCR bands were measured by densitometry (Model GS-700, Bio-Rad) and expressed relative to β-actin.

Table 1 Oligonucleotide sequences of PCR primers

Oligo	Sequence	Annealing temperature (℃)	Size (bp)
Inhibin α	5'-AGGAAGAGGAGGATGTCTCC-3'	50	823
	5'-GAGTAACCTCCATCCGAGGT-3'		
Activin βA	5'-CTGAACGCGATCAGAAAGCT-3'	54	1014
	5'-TCCTCCACGATCATGTTCTG-3'		
Activin βB	5'-TTCGCCGAGACAGATGGCCT-3'	59	765
	5'-AATCATGTTGGGCACGTCCC-3'		
ActR IA	5'-GCCCAAGGTCAACCCCAAACTCT-3'	60	265
	5'-GGATTTTCCTTTAGTGGGCAGCT-3'		
ActR IB	5'-ACCAGCTGCCTCCAGGCCAACTA-3'	62	245
	5'-GTGCTCAGGCTCCTTGAGGTGAC-3'		
ActR IIA	5'-ACCAGTGTTGATGTGGATCTT-3'	60	456
	5'-TACAGGTCCATCTGCAGCAGT-3'		
ActR IIB	5'-CATGGAACGAACTGTGTCAT-3'	62	730
	5'-AGATCCACTGAGTCTGGAGA-3'		
Smad2	5'-TAGGTGGGGAAGTTTTTGCT-3'	60	410
	5'-TTTGCATGGGACTTGATTGG-3'		
Smad4	5'-AAGGTGAAGGTGATGTTTG-3'	60	509
	5'-TAAGGGCCCCAACGGTAAA-3'		
Smad7	5'-GTGGGGAGGCTCTACTGTGTC-3'	58	477
	5'-GGCTACCGGCTGTTGAAGATG-3'		
$p21^{CIP1/WAF1}$	5′-CCTCTTCGGCCCAGTGGAC-3′	60	369
	5'-CCGTTTTCGACCCTGAGAG-3'		
β-actin	5'-TACCTCATGAAGATCCTCA-3	50	267
	5'-TTCGTGGATGCCACAGGAC-3'		

ActR: activin receptor.

Statistical analysis

Values were expressed as mean ± SD. Student's *t* test was used to evaluate differences between control and activin A-treated samples. $^aP < 0.05$ and $^bP < 0.01$ were considered statistically significant.

RESULTS

Effects of activin A on cell proliferation

Ten human gastric cancer cell lines were treated with various concentrations of activin A (10-100 ng/mL) for 24,

Table 2 Effects of activin A on cell proliferation in human gastric cancer cell lines (mean ± SD, ng/mL)

Times	24 h				48 h				72 h			
Activin A	0	10	50	100 ng/mL	0	10	50	100	0	10	50	100
AGS	100	92.7 ± 5.1	93.6 ± 4.3	93.6 ± 4.2	100	93.6 ± 2.9	94.1 ± 3.1	92.9 ± 3.9	100	94.0 ± 3.9	94.2 ± 3.7	91.7 ± 3.8
KATO III	100	94.2 ± 3.3	92.3 ± 4.6	92.1 ± 4.8	100	93.1 ± 3.6	89.8 ± 4.2	89.9 ± 3.7	100	91.1 ± 4.0	85.6 ± 4.2	84.1 ± 6.8
SNU-1	100	94.2 ± 2.6	94.0 ± 2.8	94.5 ± 2.9	100	94.6 ± 2.0	93.4 ± 5.7	94.7 ± 3.3	100	94.0 ± 3.5	94.0 ± 2.8	93.9 ± 4.1
SNU-5	100	93.9 ± 0.6	94.7 ± 2.4	94.1 ± 1.8	100	94.1 ± 3.3	94.2 ± 5.6	94.8 ± 4.0	100	94.7 ± 2.6	94.0 ± 4.3	94.8 ± 4.0
SNU-16	100	87.3 ± 2.3[a]	81.7 ± 3.3[a]	77.2 ± 4.5[a]	100	87.8 ± 2.6[a]	74.3 ± 3.4[b]	61.5 ± 2.9[b]	100	81.2 ± 4.6[a]	62.2 ± 4.5[b]	45.2 ± 4.0[b]
SNU-484	100	94.3 ± 3.9	94.0 ± 3.0	92.1 ± 2.9	100	94.9 ± 1.5	92.6 ± 3.6	94.8 ± 1.8	100	93.4 ± 4.8	92.5 ± 5.5	91.8 ± 6.8
SNU-601	100	94.2 ± 2.0	94.2 ± 1.8	91.6 ± 2.3	100	94.6 ± 3.4	94.5 ± 2.1	94.0 ± 2.9	100	94.9 ± 4.7	91.9 ± 6.1	91.3 ± 6.7
SNU-638	100	91.1 ± 2.6	92.5 ± 1.1	91.5 ± 2.5	100	93.3 ± 3.7	92.6 ± 4.2	91.8 ± 4.2	100	93.9 ± 3.3	92.0 ± 3.8	91.3 ± 4.1
SNU-668	100	94.2 ± 2.8	94.3 ± 2.3	94.4 ± 3.1	100	91.7 ± 1.4	91.8 ± 1.6	92.3 ± 4.8	100	93.2 ± 1.5	94.0 ± 2.2	94.9 ± 2.3
SNU-719	100	92.8 ± 2.8	91.6 ± 4.0	91.4 ± 1.2	100	91.7 ± 2.2	92.4 ± 5.2	91.7 ± 3.4	100	91.8 ± 2.0	88.7 ± 2.8	89.2 ± 3.1

Values are the percentage for three individual experiments, each with triplicate samples. [a]$P < 0.05$ and [b]$P < 0.01$ *vs* the control values.

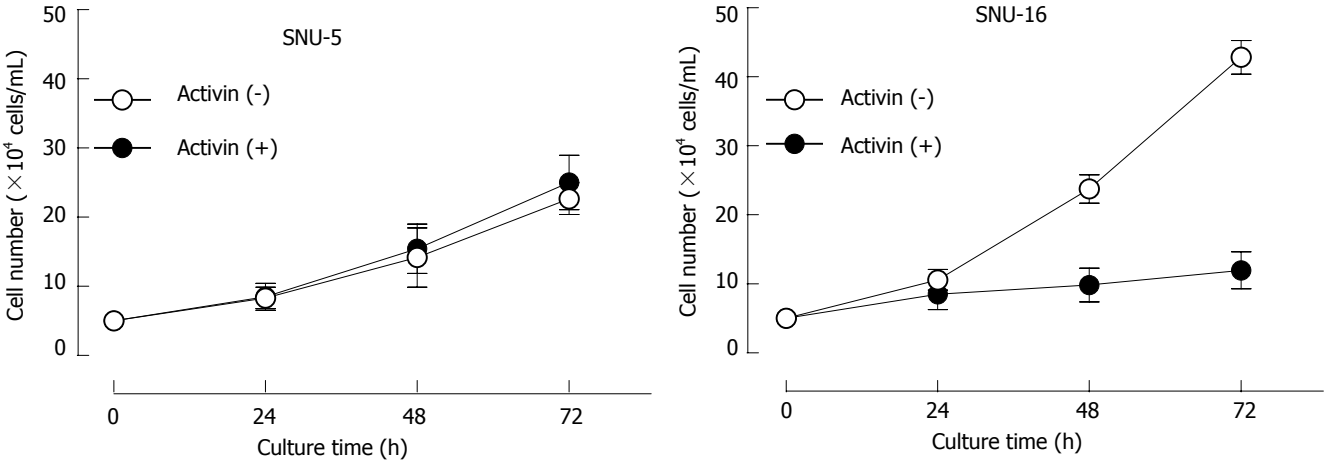


Figure 1 Effects of activin A on cell viability in SNU-5 and SNU-16 cells. Cells (5 × 10^4 cells/mL) were cultured with activin A (100 ng/mL) in a time-dependent manner and viable cells were counted with a hemocytometer. Values are the mean ± SD of three individual experiments. [b]$P < 0.01$ *vs* the control values.

48, and 72 h respectively. The effect of activin on cell proliferation was assessed by MTT assay (Table 2). Activin induced a significant decrease in SNU-16 cell proliferation in a dose- and time-dependent manner, whereas other cells showed no detectable response. To evaluate the growth inhibition of activin in SNU-5 and SNU-16 cells, the cells were cultured with 100 ng/mL of activin A for 24, 48, and 72 h respectively. The cell number was counted by a hemocytometer. Activin A treatment resulted in a significant decrease in the number of SNU-16 cells in a time-dependent manner, whereas no effect was observed in the number of SNU-5 cells (Figure 1).

Expression of inhibin/activin subunit, activin receptor, Smads, and p21$^{CIP1/WAF1}$ mRNAs

Basal expressions of inhibin/activin subunits, activin receptors, Smads, and p21$^{CIP1/WAF1}$ mRNA in human gastric cancer cell lines were investigated by RT-PCR (Figure 2). The expression of inhibin α mRNA was detectable only in SNU-638 cells whereas the activin βA mRNA was expressed in the majority of cells tested, but not in SNU-5 or SNU-719 cells. The activin βB was not detected in all cells. The activin receptor IA and IB mRNAs were expressed in all the cell lines tested. The primers for ActR IIA and IIB amplification were designed to locate within intracellular kinase domains of ActR IIA and IIB. The ActR IIA and IIB mRNAs were expressed in all the cell lines tested. Interestingly, significantly higher expressions of ActR IIA and IIB mRNAs were observed in SNU-16 cells when compared to other cells. The Smad2, 4, and 7 mRNAs were expressed in all cells and p21$^{CIP1/WAF1}$ mRNAs were not expressed in SNU-1, SNU-484, and SNU-668 cells.

Effects of activin A on expression of activin receptors, Smad and p21$^{CIP1/WAF1}$ genes

To detect the effects of activin on ActR I and ActR II mRNA expression in SNU-16 cells, RT-PCR analysis was performed using the cells treated with 100 ng/mL of activin A for various durations (Figure 3A). Expression of

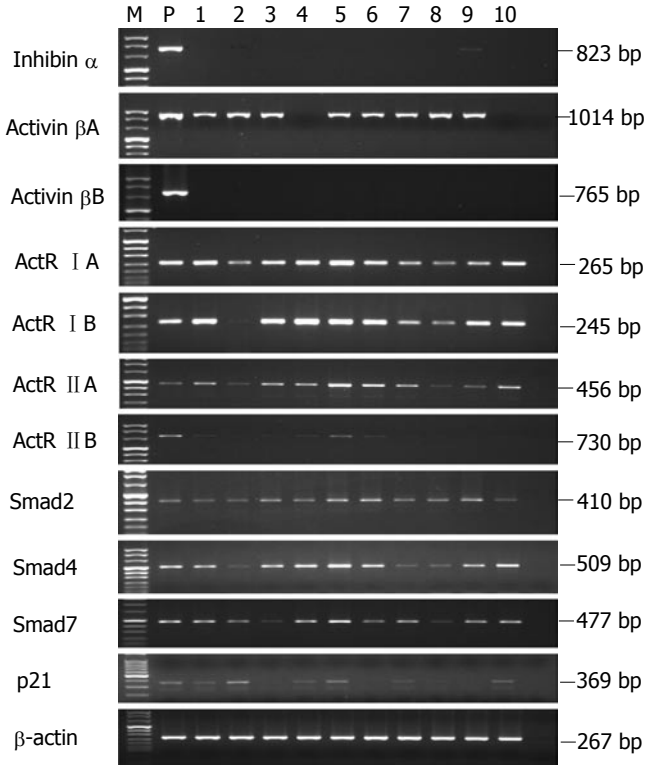


Figure 2 mRNA expressions of inhibin/activin subunits, activin receptors, Smads, and $p21^{CIP1/WAF1}$ in human gastric cancer cell lines. The mRNA levels were analyzed by RT-PCR. M: DNA size marker; P: positive control; lane **1**: AGS; lane **2**: KATO III; lane **3**: SNU-1; lane **4**: SNU-5; lane **5**: SNU-16; lane **6**: SNU-484; lane **7**: SNU-601; lane **8**: SNU-638; lane **9**: SNU-668; lane **10**: SNU-719. Positive control was used for inhibin/activin subunits (mouse ovary), activin receptor (K562), Smad and $p21^{CIP1/WAF1}$ (human keratinocytes) respectively.

ActR IA, IB, and IIA mRNA was decreased after activin A treatment, whereas ActR IIB mRNA level was increased significantly for up to 48 h in SNU-16 cells. To detect the effects of activin-signaling on Smad mRNA expression in SNU-16 cells, RT-PCR analysis was investigated using the cells treated with 100 ng/mL of activin A for various durations (Figure 3B). Although no detectable change was observed in Smad2 mRNA of SNU-16 cells, the expression of Smad4 mRNA was increased at 48 h after activin A treatment. Expression of Smad7 mRNA was increased vigorously at 24 h after activin A treatment in SNU-16 cells and decreased significantly to below initial basal level at 48 h when compared to control cells. In addition, to determine the regulation of $p21^{CIP1/WAF1}$ mRNA expression by activin in SNU-16 cells, the cells were treated with 100 ng/mL of activin A for various durations and investigated by RT-PCR (Figure 3C). Expression of $p21^{CIP1/WAF1}$ mRNA was highly increased at 72 h after activin A stimulation in SNU-16 cells.

DISCUSSION

Activin has several biological functions, including regulation of cell proliferation and inhibition of tumor cells. Activin inhibits cell growth in human prostate cancer LNCaP cells[6], human HepG2 hepatoma cells [19], and mouse B-cell hybridoma cells[20]. In this study, treatment with activin A (10-100 ng/mL) induced a significant decrease in the proliferation of SNU-16 cells in a dose-and time-dependent manner. In contrast, some human gastric cancer cell lines were relatively resistant to the growth inhibition induced by exogenous activin treatment. Interestingly, activin A (100 ng/mL) decreased the number of SNU-16 cells in a time-dependent manner. The mechanism of growth inhibition by activin in SNU-16 cells remains uncertain, but activin may act as a growth inhibitor in SNU-16 cells, and the subsequent loss of this autocrine growth inhibitory pathway may lead to the development of cancer.

Basal expressions of inhibin/activin subunits, activin receptors, Smads, and $p21^{CIP1/WAF1}$ mRNAs in the human gastric cancer cell lines were demonstrated to have differential expression patterns for inhibin α, activin βA subunits, activin receptors, and Smads, as well as the presence of $p21^{CIP1/WAF1}$ and the absence of activin βB subunits. Interestingly, significantly higher expressions of ActR IIA and IIB mRNAs were observed in SNU-16 cells when compared to other cells.

To determine the time-course of activin's effects on mRNA levels of ActRs, Smads, and $p21^{CIP1/WAF1}$, RT-PCR was performed using SNU-16 cells treated with activin A (100 ng/mL) for different time durations. Activin A decreased ActR IA, IB, and IIA mRNA levels whereas increased ActR IIB mRNA level in SNU-16 cells. Treatment with activin A (10-100 ng/mL) could not affect ActR IIA and IIB mRNA expression for 24 h in OVCAR-3 cells[21]. Activins act primarily through Smad2, possibly in partnership with Smad4, which forms heteromeric complexes with ligand-specific Smads after activation. In OVCAR-3 cells, no detectable change has been induced in Smad4 mRNA expression within 72 h of activin treatment, but the Smad2 mRNA level gradually increases and is significantly higher than that in the control at 72 h[22]. Activin–induced HepG2 liver cell apoptosis involves ActRs and Smad proteins. Overexpression of ActR IB and IIB or Smad2 and Smad4 stimulates apoptosis, whereas the dominant negative mutant forms of ActR IIB or Smad2 block activin-stimulated apoptosis. Signal transduction from the cell surface to the nuclei through Smad proteins is required for activin-induced cell death in liver cells[23]. In SNU-16 cells, no difference was observed in Smad2 mRNA levels but the Smad4 mRNA level increased for up to 48 h after activin treatment. Taken together, these results suggest that enhanced levels of ActR IIB and Smad4 mRNAs likely contribute to activin signal transduction. A novel activity of Smad7 is the inhibition of erythroid differentiation by blocking the intracellular activin signaling[14]. Smad7 expression can be induced by activin, and the overexpression of Smad7 suppresses activin-induced apoptosis in mouse B hybridomas[24]. In the present study, expression of Smad7 mRNA increased vigorously after 24 h of activin stimulation in SNU-16 cells, and then decreased at 48 h to a level significantly below the initial basal level. These results indicate that Smad7 acts as a selective blocker of activin and provides feedback regulation in the activin- signaling pathway. In human pituitary tumors with activin-inhibited proliferation, $p21^{CIP1/WAF1}$ gene expression is up-regulated in a dose-

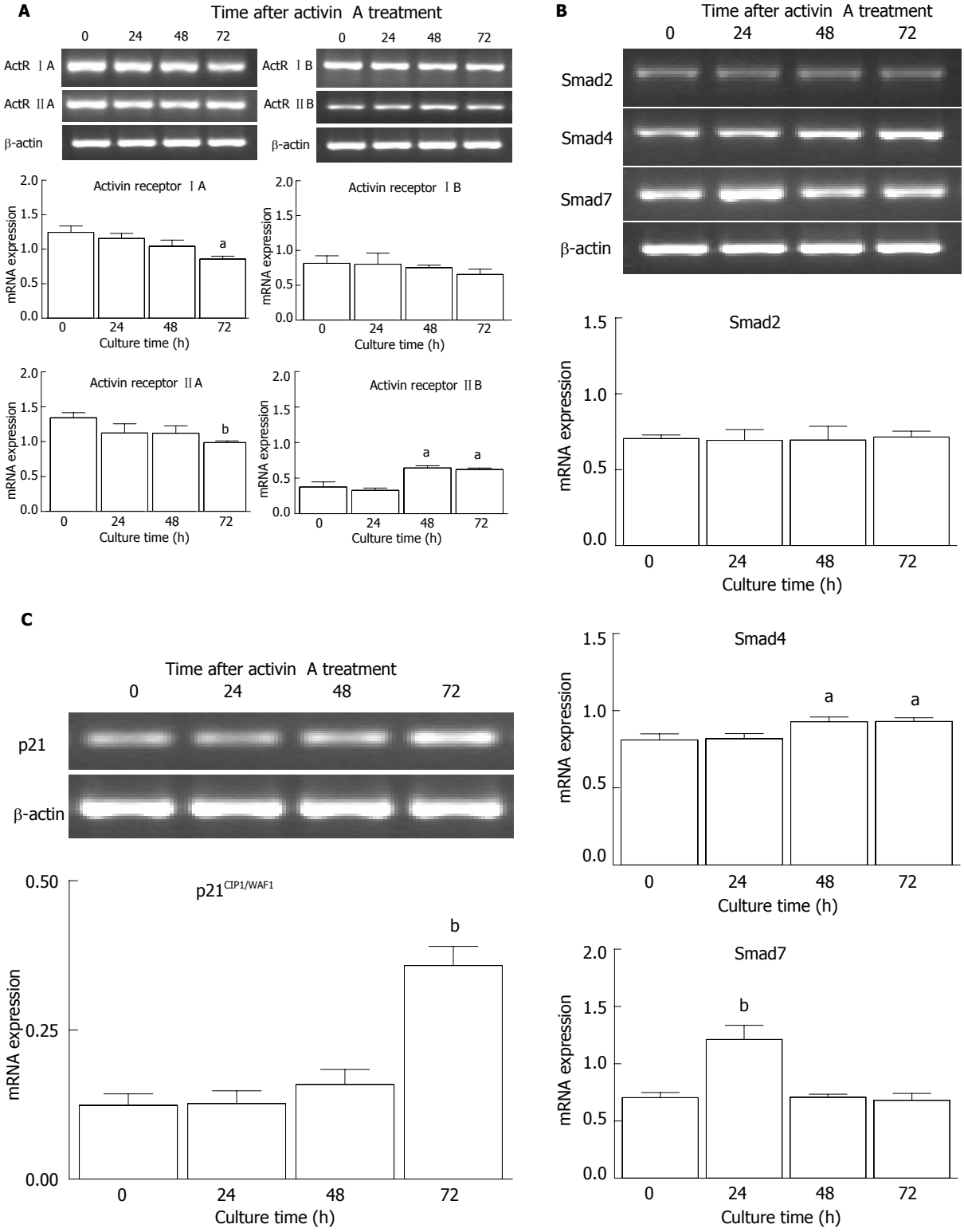


Figure 3 Effects of activin A on activin receptor mRNA expression (**A**), smad mRNA expression (**B**), and $p21^{CIP1/WAF1}$ mRNA expression (**C**) in SNU-16 cells. Cells (5×10^4 cells/mL) were cultured with activin A (100 ng/mL) in a time-dependent manner and mRNA levels were measured by RT-PCR. Values are mean ± SD of three individual experiments and reported as the ratio of activin receptors to β-actin signals, the ratio of Smad to β-actin signals, and the ratio of $p21^{CIP1/WAF1}$ to β-actin signals, respectively. $^aP < 0.05$ and $^bP < 0.01$ *vs* the control values.

dependent manner after 4 h[25]. The ectopic expression of mouse Smad7 in HS-72 cells suppresses the activin A-induced cell cycle arrest in G1 phase by abolishing the activin A-induced expression of $p21^{CIP1/WAF1}$ and the hypophosphorylation of retinoblastoma protein[25]. Compared with control cells, expression of $p21^{CIP1/WAF1}$ mRNA at 72 h after activin stimulation was higher in SNU-16 cells in this study, suggesting that the activin-induced inhibition of cell growth in SNU-16 cells is mediated through $p21^{CIP1/WAF1}$ activation.

In conclusion, inhibin/activin subunits, activin receptors, Smads, and $p21^{CIP1/WAF1}$ are expressed in human

gastric cancer cell lines. Moreover, the inhibition of cell growth by activin is regulated by the negative feedback effect of Smad7 on the activin signaling pathway, and is mediated through $p21^{CIP1/WAF1}$ activation in SNU-16 cells. Further investigation is needed to determine the molecular mechanisms of activin's inhibitory effects on cell growth, apoptosis, and cancer progression.

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VIRAL HEPATITIS

Inhibition of hepatitis B virus DNA replicative intermediate forms by recombinant interferon-γ

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Abstract

AIM: To evaluate the *in vitro* anti-HBV activity of recombinant human IFN-γ, alone and in combination with lamivudine.

METHODS: A recombinant baculovirus-HBV/HepG2 culture system was developed which could support productive HBV infection *in vitro*. Expression of HBsAg and HBeAg in infected HepG2 culture medium was detected by commercial enzyme immunoassays. HBV DNA replication intermediates were detected in infected cells by Southern hybridization and viral DNA load was determined by dot hybridization.

RESULTS: IFN-γ at 0.1 to 5 μg/L efficiently down regulated HBsAg expression in transduced HepG2 cells. At 5 μg/L, IFN-γ also suppressed HBV DNA replication in these cells. While treatment with a combination of lamivudine and IFN-γ showed no additive effect, sequential treatment first with lamivudine and then IFN-γ was found to be promising. In this culture system the best HBV suppression was observed with a pulse of 2 μmol/L lamivudine for two days, followed by 1 μg/L IFN-γ for another four days. Compared to treatment with lamivudine alone, the sequential use of 0.2 μmol/L lamivudine for two days, followed by 5 μg/L IFN-γ for six days showed a 72% reduction in HBV cccDNA pool.

CONCLUSION: This *in vitro* study warrants further evaluation of a combination of IFN-γ and lamivudine, especially in IFN-α non-responder chronic hepatitis B patients. A reduced duration of lamivudine treatment would also restrict the emergence of drug-resistant HBV mutants.

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Key words: Hepatitis B virus (HBV); Lamivudine; Interferon-γ; Replicative intermediates; cccDNA

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INTRODUCTION

Hepatitis B virus (HBV) infection is widespread in humans, with an estimated 400 million persons infected globally and an annual death rate of 1.2 million[1]. HBV is a small DNA virus with a 3.2 kb genome and belongs to the family *Hepadnaviridae* which also includes related hepatotrophic viruses from other animal species[2]. This partially double stranded genome contains four overlapping open reading frames (ORFs) that encode the surface (S, including the pre-S regions), core (C, including the pre-core region), polymerase (P), and X proteins. The HBV DNA genome replicates by a unique mechanism that involves an RNA intermediate, called the pregenomic RNA (pgRNA). The viral relaxed circular (rc) DNA genome is released in the host cytosol, migrates to the nucleus, and is converted into a covalently closed circular DNA (cccDNA) form that serves as the template for all viral transcripts. One of the major transcripts, the pgRNA is reverse transcribed into a single-stranded DNA (ssDNA) form that is the hallmark of viral replication. This first (+) strand serves as template for the viral polymerase to further synthesize the second, comparatively short (-) strand to become a partial double stranded circle (rcDNA), a minor population of which remains as a double stranded linear DNA (dslDNA) in special cases[3].

One major hurdle in the study of HBV molecular biology, its pathogenesis and the development of new antiviral agents, is the lack of suitable *in vitro* or small animal models of chronic HBV (CHB) infection in

humans. Although the use of nucleos(t)ide analogs could potentially change the course of HBV infection due to the emergence of drug-resistant mutants, novel nucleos(t)ide analog-based drugs have opened up new frontiers and challenges in the treatment of CHB. Currently, combination chemotherapy with more than one nucleo(t)side analogs are being evaluated as a new approach towards management of CHB[4]. For many years, recombinant interferon-alpha (IFN-α) has been the only effective therapeutic agent able to provide long-term disease remission for some CHB patients. However, IFN-α treatment produces significant side effects such as fatigue, fever, muscular aches, bone marrow suppression, psychosis and autoimmune conditions[5]. Although many studies have examined a combination of IFN-α and lamivudine for HBeAg-positive CHB, published trials of combination therapy provide little support for IFN-α/lamivudine combination therapy over monotherapy[6-8].

The class II interferon, IFN-γ has been used to treat hepadnaviral infections including HBV, either alone or in combination with other IFNs[9-12]. *In vitro* studies using Hep-HB107 cell lines, have shown IFN-γ to inhibit HBV replication[13]. In pilot studies, IFN-γ was shown to be effective in suppressing HBV replication in a proportion of Caucasian patients with chronic HBV infection[14-16]. Very recently, IFN-γ associated antiviral effects have been shown in woodchuck HBV (WHBV) infected woodchuck primary hepatocytes, *in vitro* as well as *in vivo* in neonates[17]. Studies have shown that analogous to other interferons, IFN-γ alone has minimal inhibitory effects on serum HBV levels[12,15]. The emergence of HBV mutants resistant to lamivudine[18] and other novel nucleoside analogs such as adefovir dilivoxil[19], famcyclovir[20] and entecavir[21] , as well as reports of non-response to IFN-α therapy[22] further limits the use of these agents.

Therefore, in this study we evaluated the antiviral potential of IFN-γ on the kinetics of HBV genome replication, as well as its efficacy in combination with lamivudine. For this, we used a recombinant baculovirus-HBV (Bac-HBV)/HepG2 culture system, similar to that developed earlier by Delaney *et al*[23]. Our results showed that sequential treatment with lamivudine and IFN-γ leads to effective suppression of HBV gene expression and genome replication.

MATERIALS AND METHODS

Cell lines and antiviral agents

HepG2 cells were obtained from ATCC and were maintained in complete DMEM supplemented with 100 mL/L FCS, 10g/L DMSO and 5 mg/L insulin (Sigma, St. Louis, MO, USA) at 37 ℃ in a humidified incubator supplied with 50 mL/L CO_2. Infected cells were maintained in DMEM supplemented with 100 mL/L FCS, 10g/L DMSO and 50 μmol/L hydroxycortisone hemisuccinate (Sigma, St. Louis, MO, USA). Recombinant human IFN-γ expressed in *E. coli*, purified and optimized for bioactivity was kindly provided by Dr. Navin Khanna (ICGEB, New Delhi). The specific activity was 3×10^{10} U/g protein and the preparation was kept in 10 mmol/L PBS, 50 mmol/L NaCl, pH 6.0 at -20 ℃. Lamivudine (3TC) was provided by Dabur Research Laboratories, Sahibabad (U.P.), India, and was kept as a 1 mmol/L stock in DMSO at -20 ℃.

Construction of recombinant HBV-baculoviruses

A 1.6-times unit-length HBV genome (5.0 kb), constructed from the parent HBV subtype-ayw infectious clone pTKHH2 (a kind gift from Dr. Angeline Barthelomeuz, Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia) was cloned into the baculovirus transfer vector, pBacPAK9 (Clontech, CA, USA) between *Xho*I and *Bgl*II sites, opposite to the polyhedrin promoter (p*Pol*) (Figure 1). The resulting pBacHep1.6 plasmid was cotransfected with Bsu36I-digested *Autographa californica* major nuclear polyhedrosis virus (AcMNPV) DNA into the cultured Sf21 insect cell line. The transfection and recombinant baculovirus selection procedures were followed as per the manufacturer's instruction. Viral plaques in the primary screen were selected by slot blot DNA hybridization with a ^{32}P-labeled HBV DNA probe. In the secondary screening, baculoviral DNA was subjected to PCR with HBV S-ORF primers. Finally, the selected recombinant Bac-HBV plaques were screened by PCR for all the HBV open reading frames. The HBV ORF-specific PCR primer sets used are as follows: C-ORF: sense, GCGGGATCCACTGTTCAAGCCTCCAAGCT; antisense, CGGAG GCGAGGGAGTTCTTCTTCT; S-ORF: sense, GCGGGATCCGGACTGGGGACC ATGTGACGAAC; antisense, GCGAAGCTTGTTAGG GTTTAAATGTATACCC; P-ORF: sense, GAAGATCTC AATCTCGGGAATCTC; antisense, GTTCGTCACAGG GTCCCCCAGTCC; X-ORF: sense, TGCCAACTGGAT CCTTCGCGGGACGTCCT; antisense, GCGAAGCTTA AGGAAAGAAGTCAGAAGG.

A high-titre stock of selected recombinant HBV-baculoviruses was prepared from 0.5 to 1.0 litre of infected Sf21 culture supernatant. The culture supernatant was cleared at 6000 r/min for 10 min and further centrifuged in a Ti45 rotor (Beckman) at 25 000 r/min for 45 min at room temperature. A bluish-white pellet seen at the bottom of the tubes was resuspended in 3 mL PBS. This was then gently overlaid on top of a 5% (3 mL) and 40% (30 mL) sucrose cushion prepared in PBS, followed by centrifugation in an SW28 rotor (Beckman) at 18000 r/min for 30 min. A band of virus particles from the 5%-40% sucrose interface was harvested and washed with 30 mL of sterile PBS by ultracentrifugation. Finally, the virus pellet was resuspended in 1 ml of sterile PBS and stored as 200 μL aliquots at -20 ℃. A working aliquot of the virus was kept at 4 ℃. The titer of the virus so prepared was determined by plaque assay on Sf21 cells to be in the range of 10^{10} pfu/mL.

Viral transduction and drug treatment of cultured cells

HepG2 cells were seeded in 12-well plates (0.5×10^6 cells/well) or 60 mm dishes (1.5×10^6 cells/dish) one day before transduction. On the day of transduction, the medium was discarded and the cells were washed twice with PBS and once with serum-free medium. Stock recombinant Bac-HBV was diluted in serum-free medium to a final multiplicity of infection (m.o.i.) of 100 in either 150 μL

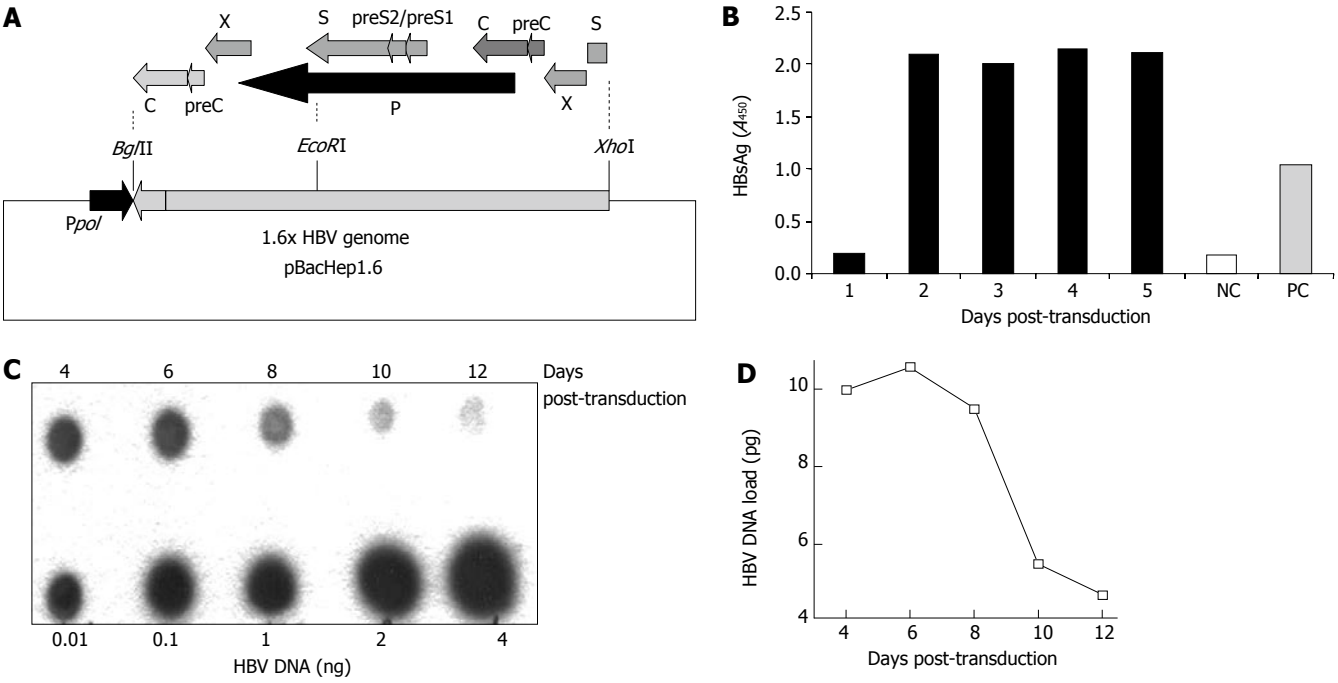


Figure 1 Construction of the recombinant Baculo-HBV virus (Bac-HBV). **A:** The recombinant transfer vector, pBacHep1.6 (10.5 kb) together with the 1.6 × genome length HBV cloned in an orientation opposite to the polyhedrin promotor (pPol) in vector pBacPAK9. The construct contains HBV-ORFs: PreS/S (1x), X (2x), P (1x), PreC/C (2x) and Enh I (2x), Enh II (2x) elements; **B:** HBsAg expression in culture medium upon transduction with 100 moi of Bac-HBV; **C:** Dot blot analysis of total HBV DNA in HepG2 cells transduced with 100 moi of Bac-HBV. Different amounts of HBV DNA were used as quantitation standards, as indicated; **D:** Densitometric analysis and quantitative representation of the results shown in **C**.

(for each well of 12-well plate) or in 300 μL (for each 60 mm dish), overlaid on the cells and incubated at 37 ℃ in a CO_2 incubator for 1-1.5 h with gentle rocking every 15 min. The infectious medium was then replaced with fresh medium and the cells incubated for the desired period with media changes every alternate day. Antiviral drugs, either alone or in combination were added on d 2 post-transduction, or as indicated for different experiments, after analysis of the baseline HBsAg expression in the culture supernatants for productive HBV infection. The drug-containing medium was replaced every alternate day throughout the study period. The doses of antiviral agents were first tested in the HepG2.2.15 cell line, prior to their use in Bac-HBV transduced HepG2 cells. Every condition was set up in at least duplicate (sometimes triplicate) wells or plates.

Analysis of HBsAg and HBeAg expressions

The secreted HBsAg and HBeAg in the culture supernatants were analyzed by the use of commercial enzyme immunoassays: HBsAg microelisa (Organon Teknika, The Netherlands) and HBeAg EIA (DiSiron SPA, Italy).

Isolation of HBV DNA from infected cells

Total and cccDNA forms of HBV DNA were isolated essentially as described elsewhere[24]. For total DNA isolation, cells were washed twice with cold PBS and lysed in 1 ml of cold lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA, 150 mmol/L NaCl, 10 g/L SDS) on ice for 10 min. The lysate was collected in an eppendorf tube, and 100 μL of proteinase K (10 g/L) was added, mixed well and incubated at 37 ℃ for 4 h. Following phenol-chloroform-isoamyl alcohol extraction, the DNA was precipitated with chilled ethanol in the presence of sodium acetate (pH 5.2) and yeast tRNA. The DNA pellet was washed with 700 mL/L ethanol, air-dried, dissolved in 30 μL of TE (pH 8.0) and stored at 4 ℃ for further use. For isolation of cccDNA, the cells were washed and lysed as above. The lysate was collected in an eppendorf tube and 250 μL of 2.5 mol/L KCl was added and mixed properly. Following incubation on ice for 30 min, the mixture was centrifuged at 13000 r/min for 30 min in a microfuge. The supernatant containing viral cccDNA was separated and subjected to phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation, as above. The cccDNA pellet was dried, dissolved in 30 μL of TE and stored at 4 ℃ for further use.

Analysis of HBV DNA

HBV DNA isolated from Bac-HBV transduced cells, either untreated or treated with antiviral drugs, was analyzed by dot blot hybridization for total viral DNA or by Southern blotting for replicative intermediates according to standard protocols. An HBV full length (3.2 kb) linear DNA was radiolabeled with 32P dCTP, using a nick-translation kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions, purified through a Sephadex G50 column and used as a probe. The signals on dot blots and Southern blots were scanned, and the bands were quantitated by densitometry using the Kodak 1D image analysis software (Kodak Digital Science, Wauwatosa, WI, USA). The signal intensities were expressed as relative pixel values for each blot.

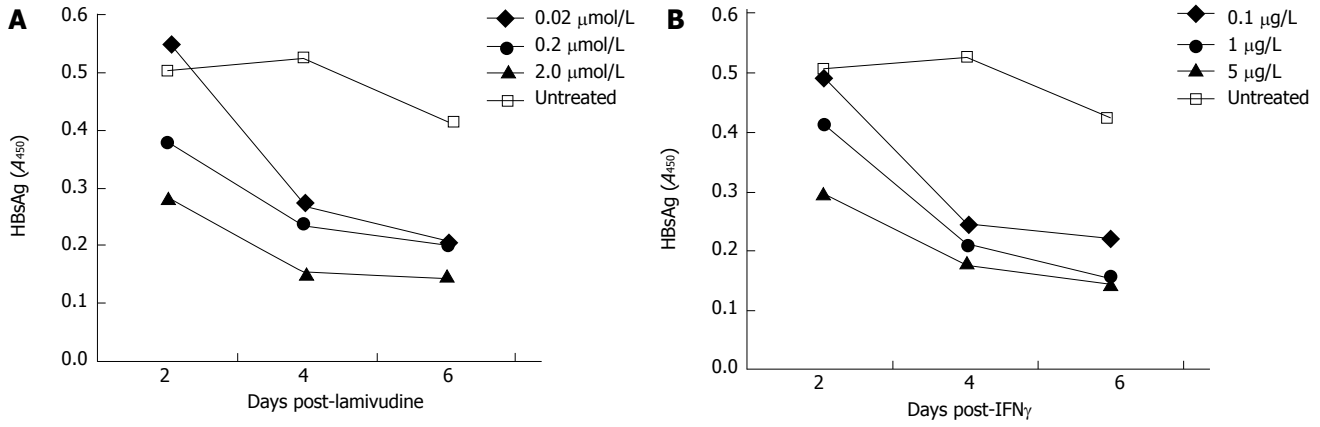


Figure 2 A time course HBsAg profile (ELISA) of transduced HepG2 cells, treated with variable doses of lamivudine and IFN-γ. **A:** Effect of lamivudine used at concentrations of 0.02, 0.2, and 2.0 μmol/L; **B:** Effect of IFN-γ used at concentrations of 0.1, 1.0 and 5.0 μg/L.

RESULTS

Generation and characterization of Bac-HBV

The subtype ayw infectious HBV clone pTKHH2 was used to isolate a 1.6 times genome-length HBV fragment as an *Xho*I-*Bgl*II fragment. This fragment included one complete copy each of the HBV P and S (including the pre-S regions) ORFs and two copies each of the C (including pre-C) and X ORFs (Figure 1A). The *Xho*I-*Bgl*II fragment was cloned into the baculovirus AcMNPV transfer vector pBacPak9 and a recombinant virus selected as described in Materials and Methods. Multiple recombinant baculoviruses were analyzed in three rounds of plaque purification. Since the HBV genome is placed in the transfer vector in an orientation opposite that of the baculovirus polyhedrin promoter, no HBV gene expression is expected in insect Sf21 cells used for recombinant baculovirus selection. However, in HepG2 cells the HBV transcription regulatory elements would be active and expression would be controlled by endogenous HBV promoters/enhancers. The Bac-HBV isolate A3-20 was finally selected for further experiments. The efficiency of transduction of HepG2 cells by Bac-HBV was estimated by HBsAg expression and total HBV DNA synthesis in transduced cells. Following transduction, we observed HBsAg synthesis (Figure 1B) and HBV DNA accumulation (Figure 1C) in HepG2 cells. High levels of HBsAg were observed in the culture medium starting at d 2 post-transduction and these levels remained constant till d 5 (Figure 1B). While it was not possible to carry on this HBsAg accumulation experiment beyond 5 d post-transduction, other experiments wherein the culture medium was changed every alternate day showed HBsAg expression to be uniformly high for up to 10 d post-transduction (data not shown). Total HBV DNA also accumulated in transduced HepG2 cells with a peak on d 6 followed by a rapid decline by d 10 (Figure 1C). The results showed that the Bac-HBV constructed here was functional and transduction of HepG2 cells led to the expression of HBV markers.

Effect of lamivudine and IFN-γ on HBV expression and replication

Since lamivudine is already established as a drug with anti-HBV properties, we tested its inhibition of the Bac-HBV system. In the range of 0.02 to 2 μmol/L lamivudine effectively reduced HBsAg levels in the culture medium of treated cells, compared to untreated cells (Figure 2A). A reproducible dose response was observed wherein with increasing drug concentration lower levels of HBsAg were observed at each of the three sampling points, d 2, 4 and 6 post-treatment. In the same experiment we also evaluated the anti-HBV effect of IFN-γ. At concentrations of 0.1 to 5 μg/L that corresponded to 3000 to 150 000 IU/L, IFN-γ also showed a marked reduction in HBsAg levels compared to untreated cells (Figure 2B). There was a rapid decline in HBsAg levels in the d 2 to d 4 period following IFN-γ treatment. We also tested whether cells pre-treated with IFN-γ and then transduced with Bac-HBV showed a similar response. HepG2 cells were either treated with IFN-γ for 24 h prior to transduction (pre-treatment group) or were first transduced and IFN-γ treatment was then initiated 24 h later (post-treatment group). In both cases, HBsAg levels in culture media were estimated 4 d after IFN-γ treatment. Pre-treatment with IFN-γ (0.01 to 10 μg/L) was found to have no significant effect on HBsAg expression, while treatment with IFN-γ after transduction was found to inhibit HBsAg expression, as observed earlier (data not shown).

To evaluate the effect of IFN-γ on HBV replication, we transduced HepG2 cells with Bac-HBV followed by treatment with 5 μg/L IFN-γ as earlier, and extracted total DNA from untreated control and treated cells at regular intervals up to ten days following IFN-γ treatment. The replicative forms of HBV were separated, identified by Southern blotting and the signals quantitated by densitometry. The results are presented in Figure 3. While no significant effect of IFN-γ was observed on the relaxed circle (RC) and double-stranded linear (DSL) forms of HBV DNA, IFN-γ treatment led to significant reduction in the covalently closed circular (CCC) and single-stranded (SS) forms of HBV DNA. The latter forms appear late in infection and are indicative of viral DNA replication as seen from their appearance around d 6 post-treatment (or d 7 of Bac-HBV transduction). While the cccDNA remains high once replication has taken place, the ssDNA form is the replicative intermediate that increases transiently and disappears at late time points. Both these

forms were suppressed around 75%-80% by IFN-γ. Overall, the kinetic study of HBV replication in transduced HepG2 cells demonstrated a time dependent, sequential synthesis of the different replicative intermediates and their inhibition by IFN-γ.

Effect of combination of lamivudine and IFN-γ on HBV expression and replication

Our *in vitro* data on lamivudine and IFN-γ monotherapy showed the effective doses of the two agents to be 2.0 μmol/L and 5 μg/L, respectively scheduled for six days (Figure 2). Comparable antiviral effects were observed at these doses. We hypothesized that a combination of lower doses of these two agents would efficiently downregulate HBV gene expression and DNA replication. If this were the case, it would be significant in a clinical setting in reducing the time of lamivudine treatment and the possibility of developing drug resistance. To test this hypothesis, we designed an *in vitro* treatment schedule of Bac-HBV transduced HepG2 cells with mixed doses of lamivudine and IFN-γ for a period of six days.

We tested two groups, one with IFN-γ at 1 μg/L and 0.02 μmol/L, 0.2 μmol/L or 2 μmol/L lamivudine, and the other group with lamivudine at 0.2 μmol/L and 0.1 μg/L, 1 μg/L or 5 μg/L of IFN-γ. Following treatment of transduced cells with these combinations, HBsAg expression levels and HBV DNA load were estimated at d 2, 4 and 6 post-treatment. As a control in the same experiment, monotherapy with same concentrations of either lamivudine or IFN-γ was also evaluated. We observed no significant reduction in either HBsAg levels or HBV load for the combinations compared to the single drugs (data not shown).

In view of these results, we further tested viral gene expression and DNA load using a sequential approach that included treatment with lamivudine alone for two days (pulse) followed by IFN-γ alone for the next four days (chase). We designed a "low" and "high" combination of the sequential treatment schedule for a period of 6 days (Figure 4A). No significant effect on either HBsAg expression (Figure 4B) or HBV DNA load (Figure 4C) was observed in the low dose sequential treatment group, when compared to either lamivudine or IFN-γ alone. However, in the high dose group, treatment first with 2 μmol/L lamivudine for 2 d followed by 1 μg/L IFN-γ for 4 d showed a significant and reproducible reduction in HBsAg expression and HBV DNA load compared to treatment with either agent alone. Since HBeAg expression and secretion in culture medium has been correlated with viral replication, we also tested its levels following lamivudine and IFN-γ treatment. Pulsing with 0.2 μmol/L lamivudine and chasing with 5 μg/L IFN-γ led to a significant reduction in HBeAg levels, compared to either agent alone (Figure 5).

With this information, we extended our studies to evaluate the inhibitory effects of a 2 d pulse of 0.2 μmol/L lamivudine, followed by a 6 d chase with 5 μg/L IFN-γ on the cccDNA pool. Southern hybridization analysis of the HBV cccDNA, isolated from the nuclei of treated or untreated Bac-HBV transduced HepG2 cells showed about 60% suppression of cccDNA by this sequential dosing, compared to lamivudine alone (Figure 6). Compared to the untreated control, this sequential dose reduced cccDNA levels by about 72%; under similar conditions, lamivudine alone reduced cccDNA forms by about 30%. Based on our *in vitro* studies, a sequential therapy with lamivudine given as a pulse of 0.2 μmol/L for the first two days and chased with 5 μg/L IFN-γ for another six days was found to efficiently suppress HBV infection.

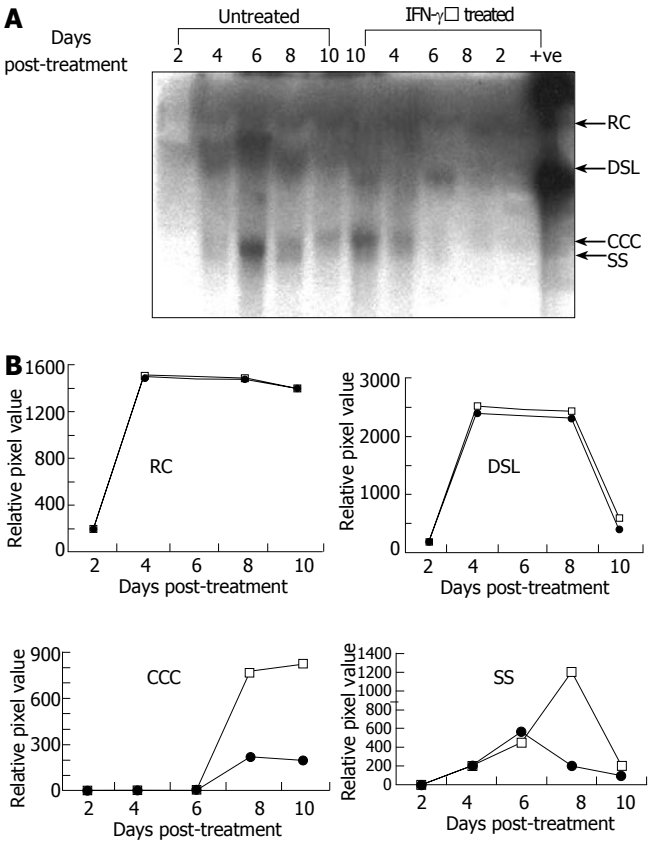


Figure 3 Representative Southern blot (**A**) and quantitative (densitometry) analysis (**B**) of effect of IFN-γ (5.0 μg/L) treatment on HBV DNA replication in transduced HepG2 cells. Synthesis of viral replicative intermediates: relaxed coil (RC), double stranded linear (DSL), covalently closed circular (CCC), and single stranded (SS) DNA forms in treated (•) and untreated (□) cells.

DISCUSSION

A major hurdle in the development of new anti-HBV agents is the lack of suitable *in vitro* as well as *in vivo* systems, which could accurately or closely model chronic HBV infection in humans. Since HBV does not directly infect cells in culture, several physicochemical and virus vector-based methods have been used to deliver HBV genome to establish productive infection *in vitro*[25,26]. A recombinant baculovirus-based system has been used to transfer HBV genome into cultured liver cell lines[23] and to test the efficacy of antiviral agents[27]. There are multiple features that make this recombinant baculo-HBV/HepG2 culture system a useful tool over stable HBV-expressing cell lines such as HepG2.2.15[23]. It is a transient system that does not require integration of the HBV genome into the cellular DNA and thus more accurately mimics CHB in humans[23]. The HBV-baculovirus infection, even at high

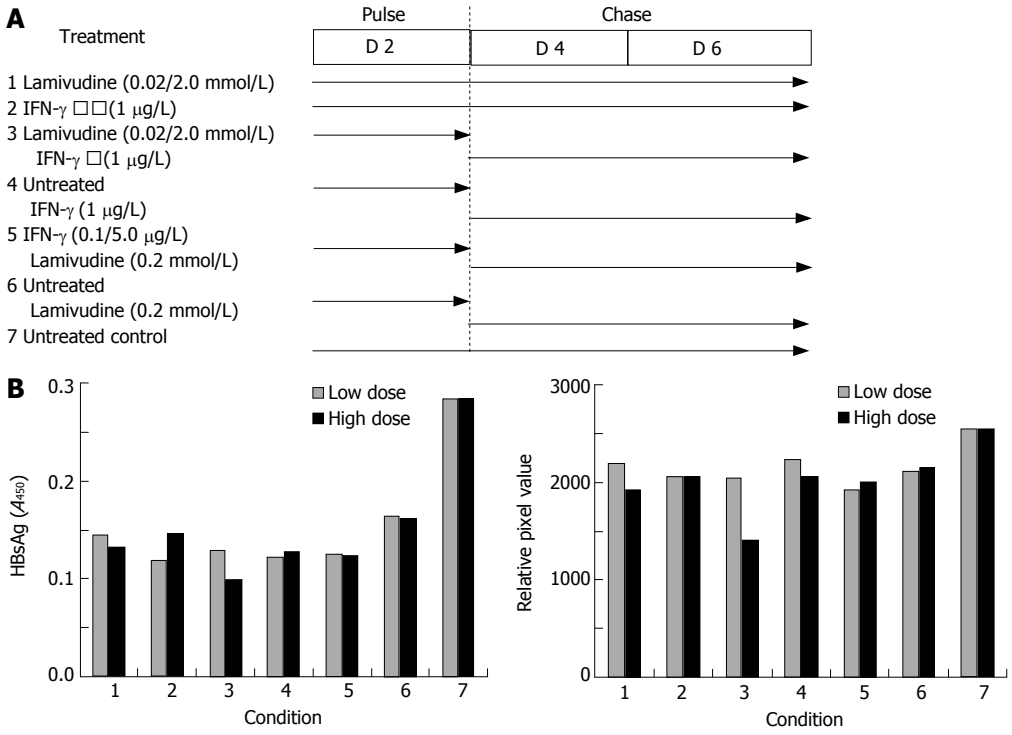


Figure 4 Effects of sequential treatment with "low/high-dose" of lamivudine and IFN-γ on HBV gene expression and total DNA load in transduced HepG2 cells. **A:** HBsAg profile; **B:** total HBV DNA upon sequential treatment of transduced cells. 1: Lamivudine (0.02 or 2.0 μmol/L) 6 d; 2: IFN-γ (1.0 μg/L) 6 d; 3: Lamivudine (0.02 or 2.0 μmol/L) 2 d + IFN-γ (1 μg/L) 4 d; 4: Untreated 2 d + IFN-γ (1.0 μg/L) 4 d; 5: IFN-γ (0.1 or 5 μg/L) 2 d + lamivudine (0.2 μmol/L) 4 d; 6: Untreated 2 d + lamivudine (0.2 μmol/L) 4 d; 7: Untreated control.

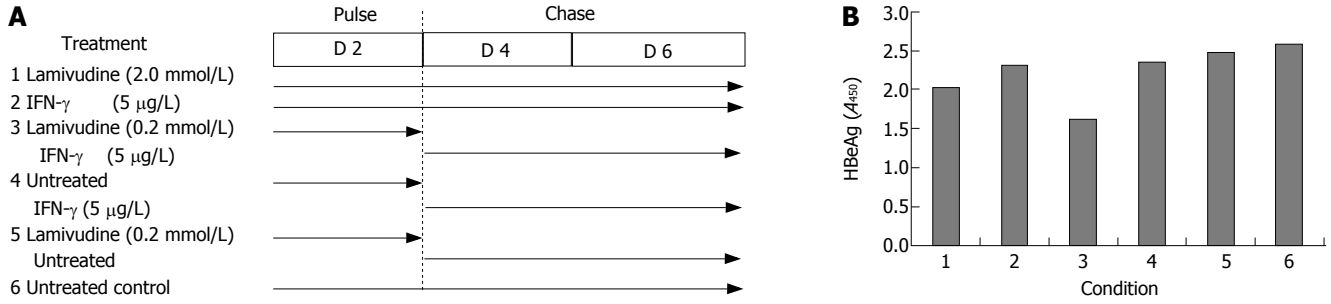


Figure 5 Effects of sequential treatment with "high-dose" of lamivudine and IFN-γ (**A**) on HBeAg expression in transduced HepG2 cells (**B**). 1: Lamivudine (2.0 μmol/L) 6 d; 2: IFN-γ (5.0 μg/L) 6 d; 3: Lamivudine (0.2 μmol/L) 2 d + IFN-γ (5 μg/L) 4 d; 4: Untreated 2 d + IFN-γ (5 μg/L) 4 d; 5: Lamivudine (0.2 μmol/L) 2 d and untreated 4 d; 6: Untreated control.

multiplicity of infection is not toxic to HepG2 cells, and HBsAg as well as HBV virions can be readily detected in the culture medium as early as 2-4 d post-infection. Many aspects of HBV biology, its pathogenicity and efficacies of antiviral drug have been studied in related hepadnaviruses like WHBV[28] and duck hepatitis B virus (DHBV)[29] that are useful models, but still different in the details of viral biology compared to HBV.

We developed our own Bac-HBV system based on published guidelines[23] and characterized it for HBsAg expression and the kinetics of HBV DNA accumulation. The analysis of HBV replicative intermediates in transduced HepG2 cells demonstrated a time-dependent, sequential synthesis of different viral DNA replicative intermediate species. The presence of high levels of dslDNA on the fourth day could be related to the onset of viral DNA replication, which coincided with the appearance of ssDNA (a hallmark of HBV replication) and kept increasing on the following days. A sudden appearance of high levels of cccDNA forms on the sixth day is likely to result from its accumulating pool

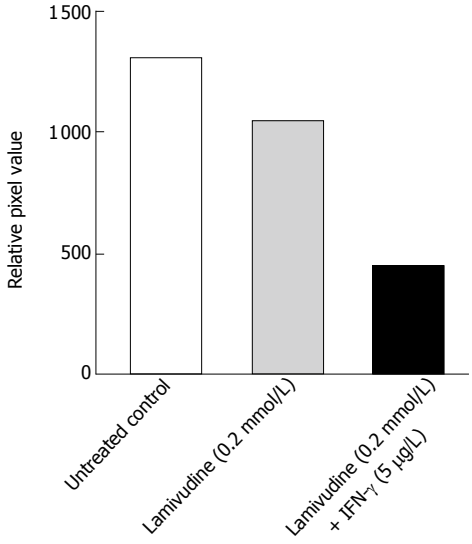


Figure 6 Effect of sequential treatment with "high-dose" of Lamivudine and IFN-γon HBV cccDNA pool in transduced HepG2 cells. The figure shows densitometric analysis of the 2 kb cccDNA form. 1: Untreated control; 2: Lamivudine (0.2 μmol/L) 8 d; 3: Lamivudine (0.2 μmol/L) 2 d + IFN-γ (5.0 μg/L) 6 d.

in the nuclei of HepG2 cells. The cccDNA is the only transcription template for all HBV RNA species, and is maintained as minichromosomes complexed with nuclear histone proteins[24,30]. This further stabilizes this minichromosomal DNA, which in turn becomes resistant to nucleases[30,31]. The cccDNA also gets recycled from the cytoplasmic-nascent rcDNAs[24]. That is probably why we observed a high cccDNA pool throughout, once it reached its equilibrium on the eighth day. The ssDNA seen at its maximum on d 8, indicated that viral genome replication was at its peak at that time. A sudden drop in its level by d 10 showed cessation of replication. Here, it is important to note that like ssDNA, the dslDNA also dropped on d 10 from its d 8 peak levels. The dslDNA is a byproduct of a minor HBV DNA replication strategy called illegitimate replication, already shown in DHBV models[31]. In this pathway, dslDNA is produced as a result of the failure to prime plus-strand DNA synthesis at the correct location, and is efficiently converted to cccDNA by non-homologous recombination near the two ends of the linear DNA. It is speculated that the low levels of virus expression in cells carrying out illegitimate replication might provide such cells a survival advantage during an antigen-specific cellular immune response[32].

Compared to IFN-α, IFN-γ is a potent immunostimulatory agent and is the major cytokine responsible for regulating the expression of both class I and class II antigens[33]. The classical concept that viral clearance is mediated by CTLs, which may destroy the infected hepatocytes has been challenged by recent studies that virus specific CTLs can abolish HBV gene expression, transcription and replication without killing the infected hepatocytes[34]. However, the distinction between the antiviral and cytotoxic effects of IFN-γ on HBV infected hepatocytes is not well characterized. In our *in vitro* study, IFN-γ was found to be effective in down regulating HBsAg levels at concentrations of 1.0 and 5.0 μg/L. The higher dose of IFN-γ was effective in suppressing viral replication by altering the kinetics of replicative DNA intermediates. It was effective on ssDNA only after the sixth day of treatment and the levels were reduced to 50% on the following days, clearly indicating cessation of replication. Interestingly, the cccDNA pool that was not effectively eliminated by lamivudine, was suppressed by 75%-80% by IFN-γ on the very day it appeared. This might be due to destabilization and degradation of cccDNA under high dose IFN-γ pressure. However, IFN-γ failed to suppress the rcDNA and dslDNA forms, despite continued therapy.

The three aims of any therapeutic strategy for CHB are (1) to decrease viral load to undetectable levels, (2) to decrease the rate of disease progression and, (3) to decrease the rate of emergence of drug-resistant HBV. Combination therapy has emerged as a new approach to treat CHB wherein the objective is to first bring down the viral load to the lowest possible levels with a potent nucleoside drug. This is then followed by a continued chemotherapy with another nucleoside analog or IFN-α in order to maintain or eliminate the remaining viral load[35-37]. Continuation with another nucleoside may again give rise to emergence of novel drug-resistance. Following the same strategy, we also evaluated combinations of lamivudine and IFN-γ in our *in vitro* system. This novel approach effectively reduced the cccDNA pool from the nuclei of transduced HepG2 cells.

Only about a third of individuals exposed to HBV are clinically diagnosed with symptomatic acute hepatitis[38]. This infection is identified only sporadically, usually post-factum, by accidental detection of circulating viral markers and serum HBV DNA. However, it has been shown that clinical recovery with biochemical normalization of liver function, the clearance of serum HBsAg and HBV DNA and the appearance of anti-HBs do not reflect virus eradication after an episode of hepatitis B[38-40]. HBV genomes persist in serum and liver despite the clearance of serum HBsAg and anti-HBs seroconversion during antiviral therapy for CHB[41,42]. Consistent molecular evidence of WHV replication not only in the livers but also in peripheral blood mononuclear cell (PBMC), bone marrow, spleen, lymph nodes, and, occasionally, in the thymus also supports the findings in the HBV-human situation. Overall, the results from recent studies unequivocally demonstrate that low-rate hepadnavirus replication in serologically undetectable infection progresses in lymphoid cells[43,44]. The lymphoid cells are believed to be an incessant, non-hepatocyte reservoir of the viral cccDNA pool as a result of "occult" infection. It is likely that this silent cccDNA may reactivate synthesis of viral transcripts and proteins and therefore a rebound of active viral replication. Therefore, elimination of cccDNA pool from infected cells still remains a challenge in HBV therapy. To the best of our knowledge, no drug or combinations of drugs to date, has been shown to effectively eliminate cccDNA.

In this study, using a combination of lamivudine and IFN-γ, we were able to successfully eliminate about three-fourths of the HBV cccDNA pool from transduced HepG2 cells. In our dot-blot analysis of the total HBV DNA levels, there was a little reduction in treated cells compared to untreated controls. This can be explained by the kinetics of HBV DNA replicative intermediates, discussed above. IFN-γ was only found to be capable of inhibiting ssDNA and cccDNA molecules. The ssDNA levels peaked on the eighth day and effects of IFN-γ were seen afterwards. The accumulation of cccDNA also started after the sixth day. Therefore at day 6 post-transduction, we cannot expect cccDNA in the total HBV DNA pool. On the other hand, IFN-γ had no effect on the other two forms, rcDNA and dslDNA. So, it is only the ssDNA, which could be suppressed on the sixth day of treatment. This is why the levels of total HBV DNA were found to be slightly down regulated, compared to the untreated controls. Overall, our *in vitro* observations of inhibitory effects of IFN-γ on HBV gene expression and DNA replication are in agreement with the published data from other groups[13,15,16]. The differences between such studies however, could be related to different dose designs, duration of chemotherapy and method of preparation of the IFN-γ used.

In conclusion, we have used the HBV-baculovirus/ HepG2 system to show that sequential treatment with lamivudine and IFN-γ efficiently suppressed HBV infection by down regulating viral replicative intermediates,

particularly the cccDNA pool. These *in vitro* results support further clinical evaluation and inclusion of IFN-γ with lamivudine as an effective combination therapy for chronic HBV infection. Such an approach may benefit those patients who do not respond to IFN-α. Further, a shortened time-course of lamivudine treatment could also help minimize emergence of drug-resistant mutants of HBV in clinical situations.

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T cell immune response is correlated with fibrosis and inflammatory activity in hepatitis B cirrhotics

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Abstract

AIM: To explore the relationship among interferon-γ (IFN-γ) activity, fibrogenesis, T cell immune responses and hepatic inflammatory activity.

METHODS: Peripheral blood samples from a total of 43 hepatitis B cirrhotic patients (LC) and 19 healthy controls (NC) were collected to measure their serum levels of IFN-γ, interleukin-2 (IL-2), soluble interleukin-2 receptor (sIL-2R), interleukin-10 (IL-10) and three serological markers of fibrosis including hyaluronic acid (HA), procollagen type III peptide (PIIIP), and type IV collagen were measured using a double antibody sandwich ELISA. Also, serum total bilirubin (TB) and alanine aminotransferase (ALT) were measured by routine measures.

RESULTS: The concentrations of serological markers of fibrosis in patients with active cirrhosis (ALC) were significantly higher than those in stationary liver cirrhosis (SLC) or NC groups. The levels of serological markers in HBeAg-positive patients were significantly higher than those in HBeAg-negative patients. In SLC and ALC patients, a negative linear correlation was found between IFN-γ levels and the serological markers of fibrosis. IFN-γ and IL-2 levels in the ALC group were significantly higher than those in the SLC and NC groups, but the statistical difference was not significant between the latter two. In contrast, IL-10 levels in the SLC group were significantly higher than that in the NC group, but no significant difference was found between SLC and ALC groups. The sIL-2R level was elevated gradually in all these groups, and the differences were significant. Positive linear correlations were seen between IFN-γ activity and ALT levels (r = 0.339, P < 0.05), and IL-2 activity and TB levels (r = 0.517, P < 0.05). sIL-2R expression was positively correlated with both ALT and TB levels (r = 0.324, 0.455, P < 0.05), whereas there was no statistically significant correlation between IL-10 expression and serum ALT and TB levels (r = -0.102, -0.093, P > 0.05). Finally, there was a positive correlation between IFN-γ and IL-2 levels.

CONCLUSION: T cell immune responses are correlated with fibrosis and hepatic inflammatory activity and may play an important role in liver cirrhosis.

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Key words: T cell immune responses; Interferon-γ activity; Fibrogenesis; Hepatic inflammatory activity

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INTRODUCTION

In Chinese population, infection with the hepatitis B virus is the major cause of liver injury. Inflammation stimulates hepatic stellate cells, which have been shown to be involved in fibrogenesis. Liver fibrosis is primarily due to an imbalance of proliferation and degradation of the extracellular matrix (ECM) triggered by hepatocytic necrosis and inflammation. Liver fibrosis is characterized by a frequent release of cytokines, such as transforming growth factor β1 (TGF-β1), platelets-derived growth factor (PDGF), tumor necrosis factor α (TNF-α), IL-6, interferon-γ (IFN-γ), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and IL-10[1]. Currently, the serum markers of fibrosis are hyaluronic acid (HA), procollagen type III peptide (PIIIP), and type IV collagen. These markers are used to reflect the activity of hepatic fibrotic process[2].

The relationship among immune response, viral clearance, inflammatory process, and liver fibrosis is very complicated. IFN-γ is not only an anti-fibrotic agent, but also a Th1-type cytokine, which participates in the immune response involved in fibrogenesis, as well as the inflammatory process. Thus, the role of T cell immune response in hepatic fibrosis is not clear.

The goal of this study was to characterize the relationship among T cell immune responses, inflammatory

activity and liver fibrosis. Thus, we assayed serum levels of IFN-γ and other T cell-type cytokines and serological markers of fibrosis in patients with varying degrees of liver inflammation stemming from hepatitis B cirrhotics.

MATERIALS AND METHODS

Patients

Forty-three patients with LC (18 females and 25 males with a mean age of 58.4 years) and 19 normal healthy adults (NC) (9 females and 10 males with a mean age of 51.4 years) were enrolled in this study. The patients with LC were all diagnosed by the same pathologist and classified according to WHO's histological classification of LC. Among them, six patients were HBeAg-positive. The patients were confirmed to have moderate hepatic fibrosis pathology, according to the Scheuer and Chevallier scoring system[3,4], stemming from chronic hepatitis B for 6 months to 30 years and none had received any form of antiviral therapy. Twenty-three of the cases were placed in the stationary LC (SLC) group and twenty cases in the active LC (ALC) group. Group designations were made according to the "2000 National Prevention and Treatment Plan of Viral Hepatitis", in which inflammation grades (G) were classified as G0, G1-2, G3 and G4 for no inflammation, mild, moderate and severe inflammation, respectively. SLC was defined as LC patients with normal level of aminotransferase, no obvious jaundice and no or mild inflammation according to pathologic inflammation grade. ALC was defined as LC patients with elevated aminotransferase and bilirubin, and moderate or severe inflammation according to pathologic inflammation grade. The clinical pathological features of each patient were reviewed and are shown in Table 1. Written informed consent was obtained from all subjects before study entry.

Peripheral whole blood samples were collected from patients and normal controls and serum was frozen at -70 ℃ until used.

Enzyme-linked immunosorbent assay (ELISA)

We measured the serum levels of IFN-γ, IL-2, sIL-2R and IL-10 as well as HA, PIIIP, IV-C by the double antibody sandwich ELISA. The IFN-γ assay kit was provided by the American R&D Corporation, IL-2 detecting reagents were purchased from the French Diaclone Corporation, and the kits for sIL-2R and IL-10 were obtained from Beijing Jinmei Biotech, Beijing. HA, PIIIP and collagen IV were determined using kits from Shanghai Maoyuan Biotech, Shanghai. Assays were performed following the manufacturer's instructions. Optical density of each well was determined within 30 min, using a microplate reader set at 450 nm.

Serum total bilirubin (TB) and alanine aminotransferase (ALT) were measured by routine methods.

Statistical analysis

All biochemical data were expressed as mean ± SD. SPSS PC+ software package was used to analyzed differences between groups for statistical significance using *t* tests. Differences were considered significant when *P* values were < 0.05. Additionally, Pearson's correlation coefficients were calculated.

Table 1 Grouping of patients with LC

According to state of illness		According to virus replication	
Group	*n*	Group	*n*
SLC	23	HBeAg (+)	6
ALC	20	HBeAg (-)	37

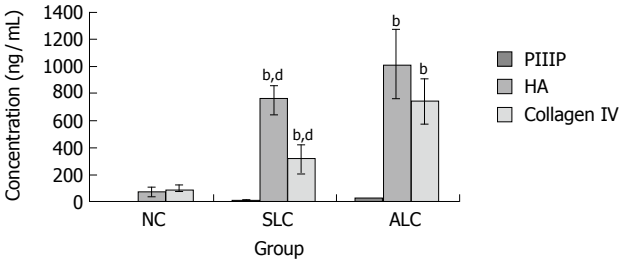


Figure 1 PIIIP, HA and type IV collagen levels in the three groups. There was a statistically significant difference between ALC and NC ($^bP < 0.01$, ALC *vs* NC) and ALC and SLC ($^dP < 0.01$, ALC *vs* SLC).

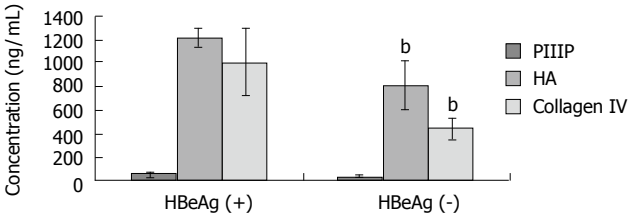


Figure 2 Relationship between serologic markers of fibrosis and HBeAg. There were statistically significant differences in levels of serologic markers of fibrosis between HbeAg (+) and HbeAg (-) groups ($^bP < 0.01$).

RESULTS

Serological markers of fibrosis and HBeAg

Figure 1 shows the concentrations of serological markers of fibrosis in NC, SLC and ALC patients [PIIIP: 2.322 ± 2.209, 16.730 ± 11.142 and 41.315 ± 20.635 ng/mL; HA: 79.247 ± 28.531, 752.870 ± 114.838 and 1 013.300 ± 252.988 ng/mL; type IV collagen: 101.974 ± 27.855, 323.661 ± 104.021 and 747.950 ± 160.929 ng/mL ($^bP < 0.01$ for all)]. The concentrations of serological markers of fibrosis in the ALC group patients were significantly higher than those in the SLC or NC groups. In addition, levels of serologic markers of fibrosis were significantly higher in HbeAg-positive patients as compared with the HBeAg-negative patients [PIIIP: 49.774 ± 21.831 *vs.* 24.665 ± 17.996 ng/mL; HA: 1 207.650 ± 79.832 *vs.* 819.866 ± 203.495 ng/mL; type IV collagen: 1 007.614 ± 286.012 *vs.* 442.105 ± 84.827 ng/mL ($^bP < 0.01$ for all, Figure 2)].

Association of IFN-γ with fibrosis

A negative linear correlation was observed between serum IFN-γ levels and serological markers of fibrosis HA, PIIIP and type IV collagen in patients with LC. The correlation

Table 2 Correlations between serum IFN-γ level and serological markers of fibrosis

	SLC			ALC		
	PIIIP	HA	Collagen IV	PIIIP	HA	Collagen IV
Correlation coefficient (*r*) with IFN-γ	-0.531[b]	-0.523[a]	-0.599[b]	-0.482[a]	-0.499[a]	-0.578[b]

[a]$P < 0.05$, [b]$P < 0.01$.

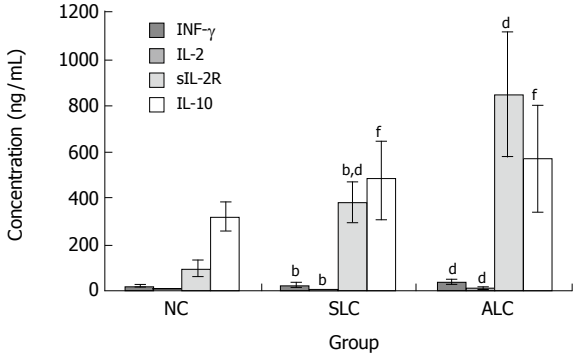


Figure 3 Serum IFN-γ, IL-2, sIL-2R and IL-10 levels in the three groups. IFN-γ and IL-2 levels were significantly higher in the ALC group compared to the SLC and NC groups ([b]$P < 0.01$, ALC *vs* SLC; [d]$P < 0.01$, ALC *vs* NC), but no obvious difference was found between the SLC and NC groups ($P > 0.05$, NC *vs* SLC). IL-10 levels were significantly higher in the SLC group compared to the NC and ALC groups ([f]$P < 0.01$, SLC *vs* NC; $P < 0.05$, SLC *vs* ALC). sIL-2R levels elevated gradually in all three groups ([b]$P < 0.01$, ALC *vs* SLC; [d]$P < 0.01$, ALC *vs* NC; [d]$P < 0.01$, NC *vs* SLC).

coefficients (*r*) of HA, PIIIP and type IV collagen with IFN-γ are listed in Table 2.

Association of T cell immune responses with inflammatory activity

Figure 3 shows IFN-γ, IL-2, sIL-2R and IL-10 levels in NC, SLC and ALC groups (IFN-γ: 21.213 ± 5.876, 23.619 ± 8.183 and 35.881 ± 9.032 pg/mL; IL-2: 5.404 ± 2.161, 6.003 ± 2.174 and 11.989 ± 7.232 pg/mL; IL-10: 320.684 ± 59.864, 480.455 ± 169.554 and 570.500 ± 230.451 pg/mL; sIL-2R: 94.917 ± 33.672, 380.571 ± 92 and 848.656 ± 268.105 pg/mL). IFN-γ and IL-2 levels in the ALC group were significantly higher than those in the SLC and NC groups ($P < 0.01$), but no significant difference between the SLC and NC groups was observed. IL-10 levels in the SLC group were significantly higher than that in the NC group ($P < 0.01$), likewise, there was no significant difference between the SLC and ALC groups. sIL-2R levels elevated gradually in all three groups, and the differences were significant ($P < 0.01$).

Positive linear correlations were found between IFN-γ activity and ALT levels ($r = 0.339$, $P < 0.05$) and IL-2 activity and TB levels ($r = 0.517$, $P < 0.05$; Table 3). sIL-2R expression was significantly positively correlated with both ALT and TB levels ($r = 0.324$, 0.455, $P < 0.05$). But the correlation between IL-10 expression and ALT and TB levels was not significant ($r = -0.102$, -0.093, $P > 0.05$). Furthermore, a positive correlation between IFN-γ and IL-2 levels ($r = 0.324$, $P < 0.05$) was observed.

Table 3 Correlations between serum IFN-γ, IL-2, sIL-2R, IL-1 levels and ALT, TB levels in LC patients

	IFN-γ	IL-2	sIL-2R	IL-10
ALT	$r = 0.339$[a]	$r = 0.119$	$r = 0.324$[a]	$r = -0.102$
TB	$r = 0.287$	$r = 0.517$[a]	$r = 0.455$[a]	$r = -0.093$
IFN-γ		$r = 0.324$[a]	$r = 0.110$	$r = 0.183$
IL-2			$r = 0.220$	$r = 0.018$
sIL-2R				$r = 0.086$

[a]$P < 0.05$.

DISCUSSION

Liver fibrosis is a common feature in chronic liver disease, which is relevant to inflammation, viral replication and T cell immune responses. T helper (Th) cells are important in T cell immune responses and are divided into Th1, Th2 and Th0 cells characterized by different cytokines they secrete. Th1 cells are characterized by IFN-γ, IL-2 and TNF-β production, whereas Th2 cells are characterized by IL-10, IL-4, IL-5 and IL-6 production. The cytokines produced by Th1 and Th2 cells can influence and restrain each other.

The present study showed that IFN-γ levels were remarkably high in the ALC group and positively correlated with ALT levels. In fact, several studies have shown that IFN-γ is associated with elevation of ALT levels in chronic hepatitis B patients[5,6]. This might be due to different immune responses in chronic *versus* acute infections.

Using IFN-γ and IL-2 levels to report the functional state of Th1 cells and IL-10 levels to report the functional state of Th2 cells in patients with LC, we found that Th1 cells predominated in the ALC group, while Th2 cells predominated in the SLC group. Recently, an imbalance of Th1/Th2 cells was found in HBV-infected chronic patients, and T cells were activated in the patients with chronic hepatitis B[7]. The majority of peripheral blood mononuclear cells shown in hepatitis B patients were Th0 cells, but the proportion of Th1 cells increased remarkably along with the hepatic inflammatory activity[8]. Th1-type cytokines levels were higher in patients with serious inflammation than those with milder inflammation. Our results also showed that there was no significant difference in IFN-γ or IL-2 levels between SLC and NC groups, thereby suggesting that Th1-type cytokines decrease with alleviation of inflammation.

An imbalance of Th1 and Th2 cells exists in patients with LC. Our results indicated a predominance of IFN-γ, IL-2 and sIL-2R in LC patients with active inflammation and a predominance of Th2-type cytokine IL-10 in ALC patients. Detection of serum levels of these cytokines might provide insight into the occurrence of liver inflammation, immune response and outcome of the patients.

IFN-γ plays a crucial role in modulating immune responses for HBV-induced cirrhosis[9-12]. Our study showed that serum IFN-γ levels were negatively correlated with serologic markers of fibrosis in ALC and SLC

patients, suggesting that IFN-γ may play a role in the fibrogenesis process in patients with LC. Many scholars hypothesize that there are two mechanisms involved in the anti-fibrotic role of IFN-γ. One role is to lower the mRNA expression level of precollagen I, III in fibroblasts. Sakaida *et al*[10] found a decrease in precollagen I, III levels and myofibroblasts in a rat liver fibrosis model, after giving the rats IFN-γ (50 000 U) daily. A second role is to inhibit activation and proliferation of hepatic stellate cells. In IFN-γ-treated animals, activation of hepatic stellate cells induced by liver injury was reduced, as shown by the decrease of proliferating hepatic stellate cells and the reduction of parenchymal area occupied by smooth muscle-specific alpha-actin (α-SMA)-positive cells. In addition, there may be some other explanations. For example, IFN-γ can cause changes in the expression of Bcl-2 and Bax genes in liver fibrosis in rats. Additionally, IFN-γ may promote myofibroblasts apoptosis and inhibit ECM synthesis[13]. Results from this study showed that levels of serologic markers of fibrosis were much higher in ALC than in SLC patients. Additionally, our results showed significantly higher levels of serologic markers of fibrosis in HBeAg-positive as compared with HBeAg-negative patients. Taken together, the data suggest that inflammation and viral replication promote the process of fibrosis.

HBV activates T cells, B cells and macrophages to express IL-2R with high competency. It has been proposed that sIL-2R as well as IL-2 might cause hepatic injury in chronic active hepatitis C[14]. sIL-2R levels decreased substantially after IFN treatment in patients with chronic hepatitis C. Consistent with our results, Sawayama *et al*[15] reported an increase of sIL-2R levels in LC patients and a positive correlation between IL-2 and ALT levels. The reason for the sIL-2R increase is still unknown, but it may be due to changes in cytokine clearance in the liver. On the other hand, activated T cells can promote the production of sIL-2R and may therefore augment serum sIL-2R levels.

A low IL-10 level could lead to an increase in pro-inflammatory cytokines and a worse prognosis. But in this study, we found non- significant correlation between IL-10 expression and ALT and TB levels (r = -0.102, -0.093, P > 0.05). In fact, an imbalance of IL-10 and IFN-γ may play an important role in promoting inflammatory reactions leading to massive liver damage[16,17], since excessive IL-10 secretion would restrain the immune response against the virus. This imbalance may thus lead to a sustained hepatitis B viral infection[5,18-20].

Because of the existence of interactions between IFN-γ and IL-2 in the immune response, we examined IL-2 levels in the LC patients. Our results showed that IL-2 levels in the ALC group were significantly higher than those in the SLC or NC groups, but IL-2 levels in the SLC and NC groups did not differ significantly. A positive correlation was found between IFN-γ and IL-2 levels as well as between IL-2 and TB levels.

In conclusion, IFN-γ ameliorates liver fibrogenesis and also participates in the cellular immune response as an important Th1-type cell, which enhances the action

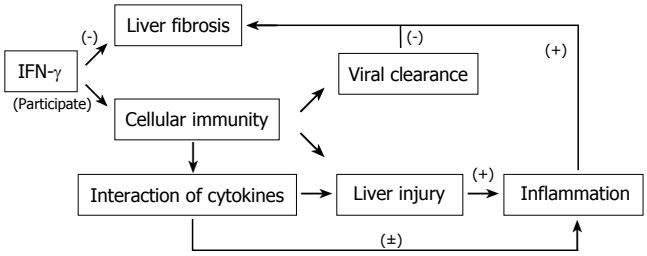


Figure 4 Simplified schemes showing the effects of IFN-γ on fibrosis. (+): upregulate; (-): downregulate.

of cytolytic T cell (CTL) to clear HBV and slows the progression of fibrosis (Figure 4). However, Th1-type cellular immunoresponses can cause liver injury during viral clearance. Inflammatory cells are recruited to the sites where hepatocytic necrosis has occurred and initiates and augments liver fibrosis. Thus, when IFN-γ is given as a treatment for LC patients during an active stage, the pro-inflammatory role of IFN-γ must be considered. At the Annual meeting of the Americans for the Study of Liver Disease (AASLD), IFN-γ has been formally recommended to be the preferred anti-fibrotic drug. Our results indicate that there still remain many problems to be solved in the course of treatment, such as IFN-γ dosage, and how to assess the efficacy of the treatment, as linked to the stage of fibrosis, inflammatory processes and serum concentration levels of IFN-γ in those patients. However, our results are important for evaluation and discussion of the feasibility of IFN-γ use in treating patients with liver cirrhosis.

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BASIC RESEARCH

Image cytometric DNA analysis of mucosal biopsies in patients with primary achalasia

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Abstract

AIM: To determine DNA aneuploidy in mucosal biopsies of achalasia patients for subsequent rapid diagnosis.

METHODS: Biopsies from the middle third of the esophagus were obtained in 15 patients with achalasia. Immunohistochemical staining was carried out with monoclonal antibodies MIB-1 for Ki67 and PAb 1801 for p53, in addition to the conventional histologic examination for dysplasia. Nuclei of fresh biopsy material were enzymatically and mechanically isolated, and the DNA content was determined with image cytometry after Feulgen staining. DNA grading of malignancy was assessed according to Boecking to determine the variability of DNA values noted around the normal diploid peak. Further indices measured included the aneuploid rate, and the 5c-, 7c- and 9c-exceeding rate.

RESULTS: The histological examination did not demonstrate dysplasia; while MIB-1 (basal) showed a positive reaction in 8/15 achalasia specimens, p53 was negative in all specimens. Image cytometric DNA analysis detected aneuploidy in 4/15 (26.7%) specimens. Samples from 15 patients with squamous cell carcinoma as well as specimens obtained exclusively 2 cm proximal to the tumor served as reference tests. All carcinomas (15/15) as well as 9 of the peritumoral samples (9/15) were aneuploid. The comparison of biopsies from achalasia patients with peritumoral and carcinoma specimens revealed statistically significant differences regarding the aneuploid rate (diploid: $P < 0.0001$; tetraploid: $P = 0.001$), grading of malignancy according to Boecking ($P < 0.0001$) and the 5c- ($P < 0.0001$), 7c- ($P < 0.0001$), and 9c- ($P = 0.0001$) exceeding rate with progredient DNA alterations in the respective order.

CONCLUSION: The finding that DNA aneuploidy was identified by image cytometry in esophageal specimens of patients with achalasia, which may be due to specific chromosomal alterations presenting as precancerous lesions in 27% of patients, leads us to conclude that image cytometry represents a valuable screening tool.

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Key words: Achalasia; Precancerous epithelial alterations; Esophageal carcinoma; Image cytometric DNA analysis; Proliferation marker Ki67; Tumor suppressor gene *p53*

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INTRODUCTION

Achalasia is a rare motility disorder of the esophagus characterized functionally and manometrically by failed relaxation of a hypertensive lower esophageal sphincter and the absence of progressive peristalsis of the esophageal body[1]. The predominant morphologic alteration is a reduction in or complete absence of ganglia cells in the myenteric plexus in combination with an inflammatory infiltrate[2-5]. Patients with achalasia are at an increased risk of developing squamous cell carcinoma of the esophagus. The incidence of carcinoma reported in the literature ranges from 0%-29%, and has led various authors to describe achalasia as a precancerous stage[6-12]. In achalasia, the carcinoma is frequently, presumably due to the presence of dysphagia, diagnosed only at an advanced stage, with a corresponding poor prognosis[8,13,14]. The detection of precancerous alterations thus remains a diagnostic challenge. In addition to the currently available optical techniques, static DNA image cytometry offers the possibility of identifying cytogenetic alterations at an early stage. In the present study, we therefore aimed to determine and investigate DNA aneuploidy in biopsy specimens from patients with primary achalasia, using a rapid diagnostic test.

MATERIALS AND METHODS

Patients

From November 1, 2003 to October 7, 2004, specimens were collected (forceps biopsies) from the middle third of the esophagus in 15 patients with achalasia in the course of screening endoscopy. The study was performed with approval of the Local Ethics Committee [number 837.347.03 (4009), 2003]. The diagnosis was confirmed in all patients enrolled in the study by esophageal manometry, as well as endoscopic and radiological examination, which enabled exclusion of the presence of secondary achalasia. Enrolled in the study were 9 male and 6 female patients with a median age of 49 (28-75) years at the time of biopsy. The median interval from the time of the initial diagnosis was 54 (1-647) mo.

Squamous cell carcinoma of the esophagus

In 15 patients with squamous cell carcinoma of the esophagus, reference specimens were obtained from the center of the carcinoma, as well as from peritumoral tissue 2 cm proximal to the tumor margin (surplus material from the surgical specimen resected in the course of esophagectomy with informed patient consent). Included in this group of patients were 1 female and 14 male patients with a median age of 64 (40-79) years.

Clinical symptoms

Clinical symptoms were assessed with the Eckardt symptom score[15] . The possible score for each symptom ranged from 0-3, depending upon the presence of dysphagia, regurgitation, and retrosternal pain occurring occasionally, daily, several times a day, or at each mealtime. An additional score was recorded for weight loss (no weight loss, < 5 kg, 5-10 kg, and >10 kg). The total possible score therefore ranged from a minimum of 0 to a maximum of 12 points.

Esophageal manometry

The investigations were carried out using a capillary perfusion system as previously described[15] . Recorded in addition to the resting tonus and relaxation of the lower esophageal sphincter were the contraction amplitudes at 5 and 10 cm above the lower esophageal sphincter after 10 barium swallows. The manometric criteria for the diagnosis of achalasia included the absence of peristalsis in the esophageal body, as well as a non-relaxing lower esophageal sphincter. Due to the fact that in one patient passage of the manometry probe into the lower esophageal sphincter was not possible, and another patient had previously undergone distal esophagus and fundus resection at an outside institution, the documentation in these patients refers to the motility of the esophageal body only.

Radiological examination

Barium swallow was performed in the patient in the supine, semisupine, and upright positions. Measurements were made of the maximum diameter of the esophageal body, as well as of the narrowest point of the gastroesophageal junction.

Therapy

Pneumatic dilatations were carried out in a total number of 10 patients (3 patients had a second, and 1 patient a third dilatation in the course of therapy). Five patients had a prior Heller myotomy in combination with anterior partial fundoplication according to Dor *et al*[16]. Nine additional patients were scheduled to undergo myotomy (in 1 of these patients re-myotomy was performed for recurrent disease).

Histology

The paraffin-embedded specimens were cut into 3-4-μm thick sections and stained with hematoxylin and eosin (H&E). The non-malignant tissue was classified as normal, inflammatorily altered, or dysplastic. The definition of invasive carcinoma was based on the detection of infiltrative growth patterns for individual malignant cells or glands.

Immunohistochemistry

Consecutive sections for immunohistochemical evaluation were cut after deparaffinization and rehydration, and 30 mL/L hydrogen peroxide was used to block endogenous peroxidase. The slides were incubated with the monoclonal antibodies MIB-1 (mAb) (1:200, Dako, Denmark) for Ki67 antigen and PAb 1801 (1:100, Becton Dickinson) for p53. After microwave pretreatment, specimens were incubated with MIB-1 and PAb for 1 h at room temperature, followed by sequential 20-min incubations with biotinylated anti-mouse IgG and peroxidase-labeled streptavidin. The staining procedure was described as positive if more than 20% of cells reacted for p53-mAb.

Image cytometric DNA analysis

A computerized image analysis system was used for DNA measurements (AutoCyte QUIC-DNA Workstation; AutoCyte, Elon College, NC, USA). The measurements were analyzed with AutoCyte QUIC-DNA software. The homogenized tissue was dissolved in 2 mL 0.01 g/L pronase solution (Pronase (lyophilyzed) Dako, Denmark) and incubated in PBS (pH 7.4). The prepared tissue was then filtered through a 50-μL pore size nylon mesh. This method enables specific staining of DNA *in situ*[17] . The possibility of DNA quantification according to the Feulgen reaction is due to the fact that the staining intensity during this reaction is in direct proportion to the DNA concentration[18] .

Approximately 250-300 test cells were measured for interactive image cytometry. The cells were selected on the screen on the basis of nuclear morphology. The DNA content of >30 reference nuclei was measured in each case to obtain accurate values at the different staining intensities for the individual slides. A reference extinction was assigned to the normal 2c-value of the reference cells. Gray levels in the microscopic image were transformed into digitalized signals and evaluated, with the System 256 allowing differentiation between gray level intervals. Measurements were made with 566-nm monochromic light. Light absorption is proportional to the DNA

content of the cell. DNA aneuploidy is assumed when cells with a DNA content of 2xG1 of the reference cell fraction are detected. In tissues without polyploidization, a threshold value of the 5c-results (5c-exceeding events) is sufficient for classification. The demonstration of 1% of cell nuclei with a DNA content of > 5c serves as proof of malignancy[19]. The following indices were calculated based on the DNA measurements: Aneuploidy (measured values outside the interval of +/- 12.5% of the value of the simple powers of 2c); aneuploid rate (percentage of aneuploid cells out of the total number of test cells); grading of malignancy according to Boecking (logarithmic transformation of the variance of DNA values on a scale from 0-3); and the 5c-, 7c- and 9c-exceeding rate (percentage of cells with ploidy values higher than 5c, 7c and 9c in relation to the total number of test cells).

Statistical analysis

The SPSS 11.0 software package (SPSS, Chicago, IL, USA: 2001) was used for data analysis. Data were expressed as median values with ranges (minimum-maximum) and as percentages. In the comparative analysis of the patient groups, the χ^2 test with Pearson's correction, as well as Fisher's exact test were employed. The Kruskal-Wallis test was used for the comparison of DNA alterations in achalasia, peritumoral, and carcinoma biopsies. Spearman' s linear regression model served to calculate potential correlations between mucosal DNA alterations or proliferation markers and clinical findings. P <0.05 was considered statistically significant.

RESULTS

The median symptom score according to Eckardt was 3 (range 1-10) points at the time of the investigation. Roentgenographic findings showed a mean maximum esophageal body diameter of 30 (20-60) mm, and a mean lumen diameter of 7 (1-10) mm at the narrowest point of the esophagogastric junction. Esophageal manometry measured a median lower esophageal sphincter resting tonus of 17.7 (5.9-28.5) mmHg (n =13). Sphincter relaxation in swallowing was absent in all patients. The contraction amplitude in the distal esophagus (mean value for 10 barium swallows) was 35.3 (6.0-65.3) mmHg at 5 cm above the lower esophageal sphincter, and 32.5 (10.0-122.8) mmHg at 10 cm.

The conventional histopathologic examination of the 15 biopsies from patients with achalasia did not demonstrate the presence of dysplasia. The immunohistochemical evaluation showed a median staining intensity with MIB-1 (basal) of 25% (0%-40%) (Figure 1), and was thus > 20% in 8 achalasia patients. Conversely, a positive reaction was noted in all patients with squamous cell carcinoma, at a median value of 70% (60%-80%). While all achalasia biopsies showed a negative reaction for p53, a positive reaction was observed for 60% of the carcinomas.

Image cytometric DNA analysis detected aneuploidy in 4 of 15 (26.7%) patients. In 9 of 15 (60%) of the peritumoral samples, aneuploidy was identified at 2 cm proximal to the tumor margin, and was present in all tumor specimens. The aneuploidy rate in achalasia was 0.9% (0%-9.4%) diploid and 0 (0%-2.4%) tetraploid. In the peritumoral tissue, the rate was 2.6% (0%-15.0%) diploid and 0 (0%-4.5%) tetraploid, compared to 12.5% (1.2%-48.0%) diploid and 1.4% (0%-15.0%) tetraploid at the center of the carcinoma. Grading of malignancy according to Boecking (scale of 0-3) was 0.2 (range 0.1-0.8) in patients with achalasia, 0.4 (range 0.1-1.5) in peritumoral tissue, and 1.3 (range 0.6-1.9) in the tumor center. The 5c-exceeding rate was 0.3% (0%-5%) in the achalasia specimens, 1.3% (0%-9.6%) in the peritumoral samples, and 10.5% (3.1%-32.6%) in the tumor center. Corresponding to the smaller sample numbers due to overlapping, the 7c- and 9c-exceeding rates were lower than the 5c-rate; there was a similar increase from achalasia to peritumoral samples and to carcinoma for these rates as that observed for the above factors.

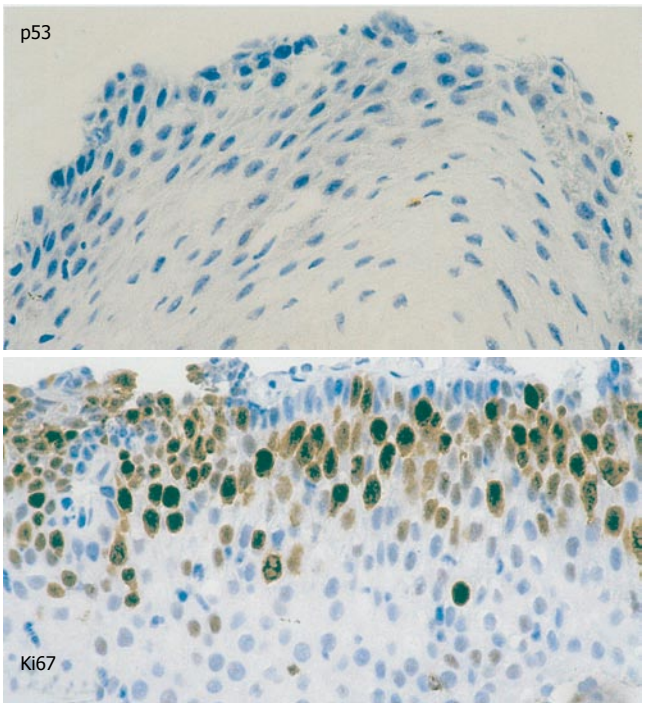


Figure 1 Immunohistochemical evaluation, staining with PAb 1801 for p53 and MIB-1 (basal) for Ki67 antigen in a patient with achalasia.

Figure 2 shows the progression of DNA aneuploidy on the basis of nuclear morphology with the respective histogram for achalasia samples (A), in peritumoral tissue 2 cm proximal to the tumor border (B), and in the tumor center of the squamous cell carcinoma (C). The comparison of biopsies from patients with achalasia, and the peritumoral as well as the carcinoma specimens employing the Kruskal-Wallis test demonstrated statistically significant differences for the aneuploidy rate (diploid: P < 0.0001; tetraploid: P = 0.001), grading of malignancy according to Boecking (P < 0.0001), as well as the 5c- (P < 0.0001), 7c- (P < 0.0001), and 9c- (P < 0.0001) exceeding rates.

There was no correlation in patients with achalasia between mucosal DNA alterations or proliferation markers

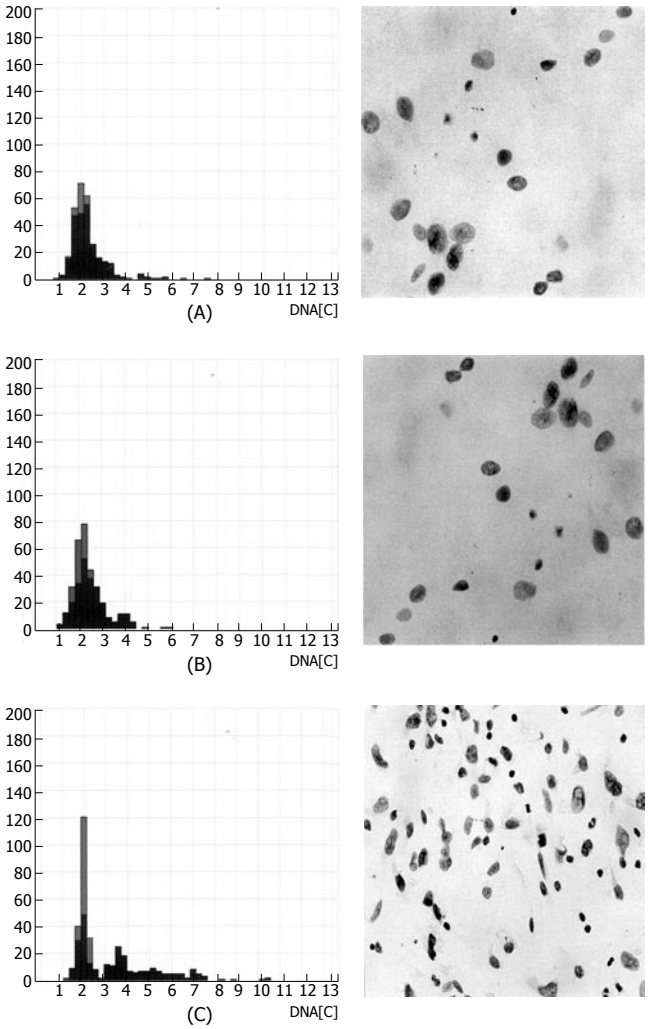


Figure 2 Image cytometric DNA analysis: histogram and nuclear morphology. (**A**) achalasia; (**B**) peritumoral tissue (esophageal carcinoma); (**C**) tumor center (esophageal carcinoma).

and the presence of clinical symptoms, the Eckardt-Score, the maximum diameter of the esophageal body, or the resting pressure of the lower esophageal sphincter ($P > 0.05$). Following allocation of the patients with achalasia to 2 groups (patients with aneuploidy: $n = 4$ and without aneuploidy: $n = 11$), a difference in the diameter of the esophageal body becomes apparent, which is relevantly larger (42.5 *vs* 30 mm) in the patients with aneuploidy; however, no differences were observed with regard to persistence of symptoms, the Eckardt-Score, and resting pressure.

DISCUSSION

DNA aneuploidy and increased cell proliferation have an important role in the pathogenesis of esophageal carcinoma. The prognostic significance of aneuploidy has been described for squamous cell carcinoma and adenocarcinoma of the esophagus[20-22]. Even in the absence of positive findings on conventional histopathology, Image cytometry offers the detection of early mucosal DNA alterations and therefore serves as a useful tool in the selection of a defined group of patients at risk for malignancy. One of the advantages of image-directed cytometry over flow cytometry is that it enables measurement of the integrated optical staining density per specimen in a digitalized image, allowing the visual assessment of the individual measured objects with the exclusion of artifacts. The presence of aneuploidy in 27% of our patients with achalasia is in accordance with the increased malignancy rate known for this disorder, and provides the possibility of early tumor detection. In addition, the method used in this study, which is based on the modification of enzymatic nuclear isolation with pronase, represents a useful rapid diagnostic test for the identification of DNA alterations.

Conventional methods of nuclear isolation for Feulgen staining and subsequent image cytometric DNA diagnosis have primarily been performed in paraffin-embedded samples and were characterized by both a minimal yield of nuclei and low staining intensity for the small number of available nuclei[23,24]. The disadvantage of paraffin-embedded or formalin-fixed samples is that unavoidable artifacts and alterations in the cell structure occurring during nuclear isolation lead to falsification of the results[25,26]. The described modification of the pronase method for nuclear isolation from non-formalin-fixed native specimens used in this study was shown to be readily reproducible. This was due to the high number of cell nuclei, which were markedly in excess of the required number of 250 study and 30 reference nuclei, as well as to negligible clumping and clearly identifiable nuclear structures in the absence of artifacts. The evaluation of the samples prepared according to the described method in combination with additionally performed grading of malignancy proved to be highly reliable.

It has been speculated that precancerous alterations in achalasia develop as sequelae of a chronic inflammatory reaction of the esophageal squamous epithelium due to the retention and stasis of food and secretions. Conventional histomorphology has identified various mucosal alterations, including diffuse squamous epithelial hyperplasia, lymphocytic esophagitis, lymphocytic inflammatory alterations of the lamina propria mucosa, and the submucosa with prominent germinal centers, as well as periductal or glandular inflammatory reactions with complete loss of submucosal glandular structures[27,28]. The development of a multistep process from squamous epithelial hyperplasia to dysplasia, culminating in the manifestation of carcinoma may be hypothesized.

DNA aneuploidy in achalasia has previously been investigated only by one study, which observed a markedly lower number of chromosomal alterations (10%) than that found in the current investigation. As reported by Bektas *et al*[29], there were no pathologic findings in the control group of his study (0/18 controls). However, the method used by Bektas *et al*[29] was flow cytometry, whose limitations have been described elsewhere in this paper. The role of aneuploidy with regard to the neoplastic progression in achalasia-associated esophageal squamous cell carcinoma has been described in a case study carried out by Porschen *et al*[30]. In their patient, aneuploidy was also found in the mucosa adjacent to the

carcinoma, a phenomenon which was likewise observed in the reference specimens from the carcinoma patients of our study. This finding may correspond to the model of epithelial lateral spread in squamous cell carcinoma leading to the development of multiple adjacent carcinomas of monoclonal origin described by Brieger *et al*[31]. Nevertheless, the time of occurrence, cellular interactions and induction by endogenous and exogenous noxae, and the distribution pattern of precancerous alterations in achalasia are currently not known.

Although dysplasia was not identified in the achalasia patients of this study - in correlation with DNA alterations - a positive reaction for Ki67 as a marker of increased proliferation was nevertheless found. Fujii *et al*[32] noted an increased number of Ki67-positive cells in the non-malignant epithelium of 4 patients with achalasia and esophageal carcinoma. While mutations of the p53 tumor suppressor gene are known to be of crucial importance for the pathogenesis of esophageal squamous cell carcinoma, the role of this gene in the development of achalasia-associated carcinoma is still under discussion. Conversely, immunohistochemical studies with p21, p16 and EGF (epidermal growth factor) have shown that dysplastic epithelium found in esophageal carcinoma represents a borderline alteration between hyperplasia and the carcinoma *in situ* in achalasia[33].

DNA aneuploidy in patients with achalasia can be identified in 27% of cases in the absence of histomorphologically proven dysplasia using image cytometry. Marked overlapping of chromosomal alterations between achalasia biopsies with peritumoral tissue and the tumor center was found in individual patients with esophageal squamous cell carcinoma. The identification of DNA aneuploidy may thus be of major importance in the early diagnosis of carcinoma in achalasia. Further long-term studies in patients with aneuploidy are needed to answer the question as to the cost-benefit effectiveness of this procedure. Should the decision favor of the use of image cytometry as an early diagnostic tool be made at this early stage, the described method could serve as clinically relevant rapid diagnostic test.

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BASIC RESEARCH

γ-aminobutyric acid secreted from islet β-cells modulates exocrine secretion in rat pancreas

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Abstract

AIM: To investigate the role of endogenous γ-amino-butyric acid (GABA) in pancreatic exocrine secretion.

METHODS: The isolated, vascularly perfused rat pancreas was employed in this study to eliminate the possible influences of extrinsic nerves and hormones. Cholecystokinin (CCK; 10 pmol/L) was intra-arterially given to stimulate exocrine secretion of the pancreas.

RESULTS: Glutamine, a major precursor of GABA, which was given intra-arterially at concentrations of 1, 4 and 10 mmol/L, dose-dependently elevated the CCK-stimulated secretions of fluid and amylase in the normal pancreas. Bicuculline (10 μmol/L), a $GABA_A$ receptor antagonist, blocked the enhancing effect of glutamine (4 mmol/L) on the CCK-stimulated exocrine secretions. Glutamine, at concentrations of 1, 4 and 10 mmol/L, dose-dependently increased the GABA concentration in portal effluent of the normal pancreas. The effects of glutamine on the CCK-stimulated exocrine secretion as well as the GABA secretion were markedly reduced in the streptozotocin-treated pancreas.

CONCLUSION: GABA could be secreted from β-cells into the islet-acinar portal system after administration of glutainine, and could enhance the CCK-stimulated exocrine secretion through $GABA_A$ receptors. Thus, GABA in islet β-cells is a hormone modulating pancreatic exocrine secretion.

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Key words: GABA; $GABA_A$ receptor; GABA secretion; Cholecystokinin; Islet of langerhans; Pancreas

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INTRODUCTION

γ-aminobutyric acid (GABA), a well-known inhibitory neurotransmitter, is also present in islet β-cells of the pancreas at a very high concentration comparable to that in the brain[1,2]. Glutamic acid decarboxylase (GAD) and GABA transaminase also co-exist in islet β-cells[3-5]. GABA has been known as a modulator of the endocrine pancreas. It has been reported that GABA and its agonist inhibit release of somatostatin[6] and glucagon[7] whereas it stimulates release of insulin[8]. The GABA concentration and the GAD activity are very high in insulinoma tissue[1] while the GAD level is very low in the pancreatic tissue with insulin-dependent diabetes mellitus[9].

In the pancreas, a substance secreted from islet cells reaches acinar cells at a very high concentration through the intra-pancreatic circulation, the islet-acinar portal system, and then gives a vast influence on exocrine function[10,11]. Thus, we hypothesized that GABA might also play a role in pancreatic exocrine function if it would be secreted from islet β-cells into the portal system. There are some reports suggesting that islet β-cells may secrete GABA and that GABA may affect pancreatic exocrine function. The pancreatic β-TC6 cells, a murine β-cell line developed from insulinoma, secrete GABA in response to glucose[12]. Cultured rat β-cells also secrete GABA into the medium in response to glutamine[13]. High affinity binding sites of GABA have been determined in pancreatic exocrine cells[14]. We have previously reported that exogenous GABA enhances CCK-stimulated exocrine secretion while it has not any influence on spontaneous or secretin-stimulated exocrine secretion of the isolated rat pancreas[15].

Thus, this study was aimed to investigate whether GABA in islet β-cells acts as a hormone to modify pancreatic exocrine secretion. Secretion of GABA from islet β-cells and effects of endogenous GABA on pancreatic exocrine secretion were determined in this

study. Bicuculline was used to interfere with the GABA action by blocking $GABA_A$ receptors. Streptozotocin was employed to destroy islet β-cells containing GABA. The totally isolated, vascularly perfused pancreas model was used in this study to eliminate the possible influences of extrinsic nerves and hormones on exocrine secretion.

MATERIALS AND METHODS

Preparation of totally isolated and vascularly perfused pancreas

Male Sprague-Dawley rats, weighing 250-300 g, were anaesthetized with an intra-peritoneal injection of 25 g/L urethan (Sigma, St. Louis, MO) at a dose of 7 mL/kg of body mass after 24 h fasting with free access to water. Rats were sacrificed by an intravenous overdose of urethan after isolation of the pancreas. The isolated, vascularly perfused rat pancreas was prepared according to a method described previously[16,17]. In brief, the abdominal aorta was carefully dissected and cannulated with PE-50 tubing (Clay Adams, Parsippany, NJ) just above the celiac artery, and then tightly ligated below the superior mesenteric artery. The pancreatic duct was cannulated at the duodenal end with PE-10 tubing (Clay Adams). The portal vein was also cannulated with Tygon microbore tubing (Fisher Scientific, Pittsburgh, PA) to drain perfusate. The isolated pancreas was perfused with modified Krebs-Henseleit solution (pH 7.4, 305 mosmol/kg water) through the celiac and superior mesenteric arteries at a flow rate of 1.2 mL/min by using a multistaltic pump (Buchler, Kansas, MO). The perfusate contained 1 g/L bovine serum albumin (Sigma), 3 g/L Dextran T-70 (Sigma) and 18 mmol/L glucose (Sigma), and was continuously oxygenated with 95 mL/L O_2 containing 5 mL/L CO_2. The pancreas was isolated with the duodenum, but separated from other neighboring organs and tissues, and then placed in a temperature-controlled experimental chamber at 37 ℃. The chamber was also continuously supplied with Krebs-Henseleit solution at a flow rate of 0.35 mL/min and also oxygenated. After an equilibration period of 30 min, pancreatic juice secreted in 15 min was collected throughout the whole period of the experiment. Portal effluent drained in 15 min was also collected in ice-chilled tubes, and then kept at -70 ℃ for GABA assay.

Effects of glutamine and GABA on CCK-induced exocrine secretion

Exocrine secretion of the isolated rat pancreas was stimulated by intra-arterial infusion of synthetic CCK-8 (Squibb Institute, Princeton, NJ) at a concentration of 10 pmol/L for 90 min. Glutamine (Sigma), a major precursor of GABA[14,18], was added to perfusate at a concentration of 1, 4, or 10 mmol/L from 30 min prior to the infusion of CCK until the end of the experiment. For mimicking the effects of glutamine, GABA (Sigma) at a concentration of 30 μmol/L was intra-arterially infused to the pancreas from 30 min before the CCK infusion until the end of the experiment. Bicuculline (Tocris, Baldwin, MO), a $GABA_A$ receptor antagonist[15,19], was intra-arterially infused at a concentration of 10 μmol/L from 30 min before the infusion of CCK until the end of the experiment. Effects of glutamine (4 mmol/L) on the CCK-stimulated pancreatic exocrine secretion were also observed after destruction of islet β-cells by streptozotocin (Sigma). Streptozotocin was intra-peritoneally injected at a single dose of 75 mg/kg 3 d before the experiment.

Effects of glutamine on GABA secretion

Glutamine (Sigma) was intra-arterially infused to the isolated pancreas at a concentration of 1, 4, or 10 mmol/L from 30 min prior to the infusion of CCK until the end of the experiment. The GABA concentration in portal effluent drained in 15 min was determined by an enzymatic recycling for NADPH as reported previously[1,14]. Briefly, 500 μL portal effluent of the isolated perfused rat pancreas was lyophilized. The lyophilized samples were dissolved in 80 μL GABA assay reagent (0.3 mol/L Tris-HCl buffer-pH 8.9, 10002 nkat/L GABAse, 5 mmol/L α-ketoglutarate, 0.1 g/L mercaptoethanol, 0.5 mmol/L $NADP^+$) and incubated for 30 min at 37 ℃. At the end of the reaction, 20 μL of 1.5 mol/L NaOH was added and the incubation was continued for 20 min at 60 ℃. A 20 μL aliquot was mixed with 250 μL enzyme recycling reagent (0.2 mol/L Tris-HCl buffer-pH 8.0, 5 mmol/L ketoglutarate, 1 mmol/L glucose-6-phosphate, 25 mmol/L ammonium acetate, 1 mmol/L ADP, 0.2 g/L BSA, 55,011 nkat/L glutamate dehydrogenase, 2.5 mmol/L glucose-6-phosphate dehydrogenase). The reaction was carried out at 37 ℃ for 1 h and stopped by heating at 100 ℃ for 7 min. A 90 μL aliquot was mixed with 250 μL assay reagent (0.1 mmol/L Tris-HCl buffer-pH 8.0, 1 mmol/L $NADP^+$, 0.4 mmol/L EDTA, 366.7 nkat/L 6-phosphogluconate dehydrogenase) and incubated for 30 min at 25 ℃. The final absorbance was recorded at 340 nmol/L. All chemicals were purchased from Sigma.

Pancreatic secretions of fluid and amylase

The volume flow of pancreatic juice was determined by measuring the length of pancreatic juice collected in microtube with a capacity of 3.8 μL/cm. α-amylase activity in pancreatic juice was determined by a method reported previously [20, 21].

Statistical analysis

All results were illustrated as mean ± SE. The data were analyzed using the Student's *t* test. The difference was considered significant when $P < 0.05$.

RESULTS

Effects of glutamine on CCK-stimulated exocrine secretion

The isolated, perfused rat pancreas spontaneously secreted a minute amount of juice (34.8 ± 5.8 nL/min) and amylase activity (5.80 ± 1.86 nkat/min). CCK-8, given intra-arterially at a concentration of 10 pmol/L, significantly increased ($P < 0.01$) the basal pancreatic secretions of fluid and amylase to 304.7 ± 23.5 nL/min and 82.83 ± 7.11 nkat/min, respectively. Figure 1 illustrates the effects of glutamine on the CCK-stimulated exocrine secretions of the isolated rat pancreas. Glutamine, added in the perfusate at concentrations of 1, 4 and 10 mmol/L, dose-dependently elevated the CCK-stimulated pancreatic

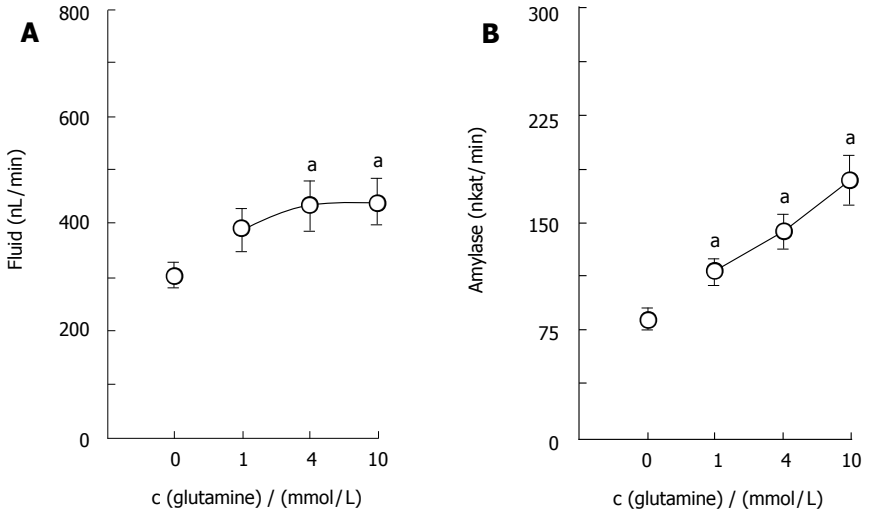


Figure 1 Dose-dependent effects of glutamine on CCK-stimulated secretions of fluid (**A**) and amylase (**B**) in the isolated, perfused rat pancreas (mean ± SE, $n = 7$). $^aP < 0.05$ *vs* without glutamine.

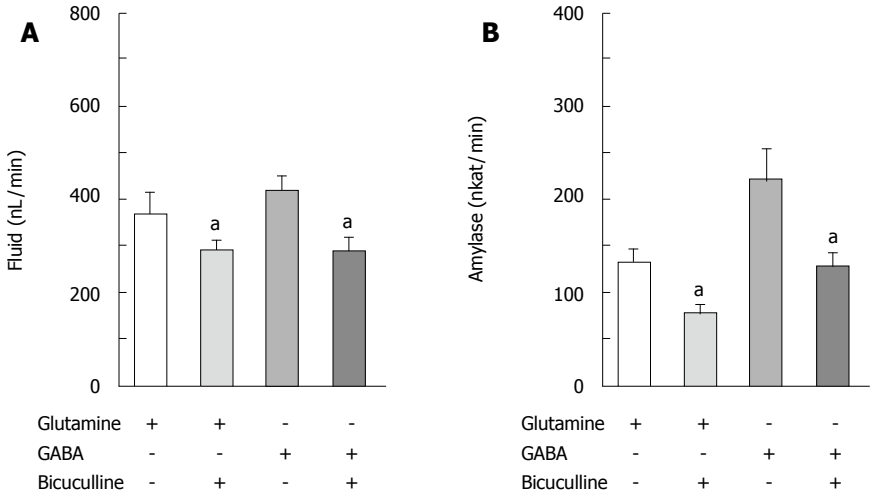


Figure 2 Effects of bicuculline on glutamine- or GABA-enhanced, CCK-stimulated secretions of fluid (**A**) and amylase (**B**) in the isolated, perfused rat pancreas (mean ± SE, $n = 7$). $^aP < 0.05$ *vs* without bicuculline.

secretions of fluid and amylase to 387.3 ± 39.0 nL/min and 115.71 ± 9.50 nkat/min, 432.3 ± 48.3 nL/min and 144.02 ± 11.88 nkat/min, 439.0 ± 45.3 nL/min and 179.18 ± 17.48 nkat/min, respectively. Figure 2 illustrates the effects of bicuculline, a $GABA_A$ receptor antagonist, on actions of glutamine and GABA in pancreatic exocrine secretions. Synthetic GABA, given intra-arterially at a concentration of 30 μmol/L, also increased ($P < 0.001$) the CCK-stimulated pancreatic secretions of fluid and amylase to 418.7 ± 31.8 nL/min and 221.82 ± 32.11 nkat/min, respectively. Bicuculline at a concentration of 10 μmol/L significantly reduced ($P < 0.001$) the glutamine (4 mmol/L)- or GABA (30 μmol/L)-enhanced, CCK-stimulated pancreatic secretions of fluid and amylase to 289.3 ± 22.0 nL/min and 78.72 ± 7.00 nkat/min or 290.2 ± 27.7 nL/min and 130.00 ± 13.41 nkat/min, respectively. The enhancing effects of glutamine (4 mmol/L) on the CCK-stimulated secretions of fluid and amylase were greatly diminished in the pancreas isolated from the streptozotocin-treated rats (Figure 3).

Effects of glutamine on GABA secretion

The mean basal concentration of GABA in portal effluent of the normal pancreas was 0.72 ± 0.08 μmol/L. Figure 4 demonstrates the dose-dependent effects of glutamine on GABA secretion in the isolated rat pancreas. When glutamine, at concentrations of 1, 4 and 10 mmol/L, was added to the perfusate containing 10 pmol/L CCK, the GABA concentrations in portal effluent increased from the basal level to the peak level of 4.72 ± 1.01 μmol/L, 8.94 ± 0.81 μmol/L and 12.11 ± 0.75 μmol/L, respectively. Figure 5 illustrates the effects of glutamine on GABA secretion in the pancreas isolated from streptozotocin-treated rats. The streptozotocin-treated pancreas spontaneously secreted GABA at a rate of 0.55 ± 0.04 μmol/L, which was significantly lower ($P < 0.05$) than that obtained in the normal pancreas. When glutamine, at 4 mmol/L, was added to the perfusate containing 10 pmol/L CCK, the GABA concentration was increased from the basal level to the peak level of 2.08 ± 0.28 μmol/L, which was also significantly lower ($P < 0.001$) than that observed in the normal pancreas.

DISCUSSION

The results of the present investigation clearly demonstrate that glutamine has an influence on exocrine secretion in the isolated, perfused rat pancreas. When glutamine was intra-arterially given at a concentration of 1, 4 or 10 mmol/L to the isolated pancreas, the CCK-

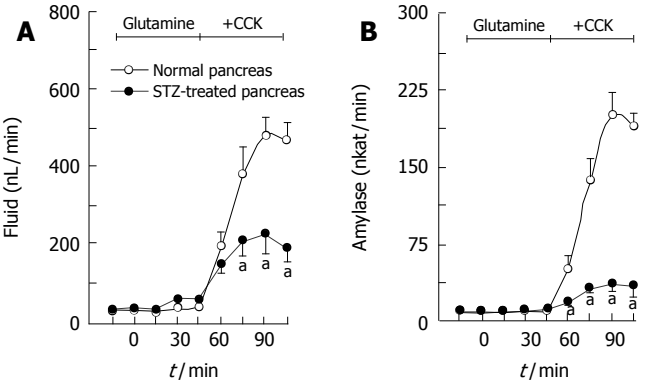


Figure 3 Effects of glutamine on CCK-stimulated secretions of fluid (**A**) and amylase (**B**) in the pancreas isolated from the streptozotocin (STZ)-treated rat (mean ± SE, $n = 7$). $^aP < 0.05$ *vs* STZ-treated pancreas.

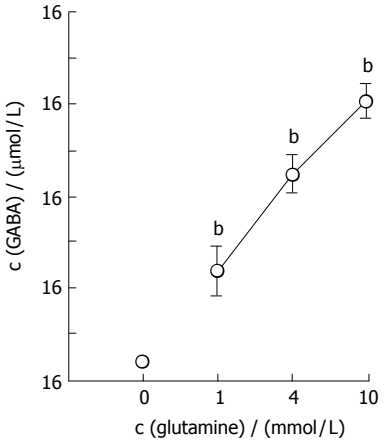


Figure 4 Dose-dependent effects of glutamine on GABA secretion in the pancreas isolated from the normal rat (mean ± SE, $n = 7$). $^bP < 0.01$ *vs* without glutamine.

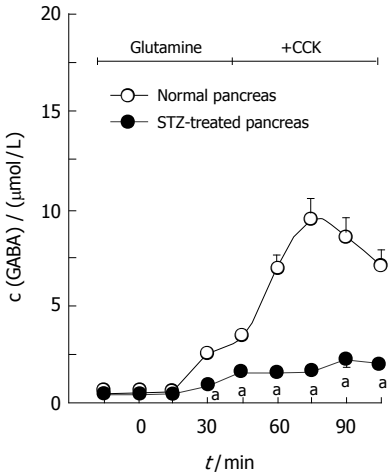


Figure 5 Effects of glutamine on GABA secretion in the pancreas isolated from the streptozotocin-treated rat (mean ± SE, $n = 7$). $^aP < 0.05$ *vs* normal pancreas.

stimulated pancreatic secretions of fluid and amylase were further elevated in a dose-dependent manner. Synthetic GABA, administered intra-arterially at a concentration of 30 μmol/L, also further increased the CCK-stimulated pancreatic secretions of fluid and amylase, as demonstrated previously[15]. Bicuculline, a $GABA_A$ receptor antagonist[19], effectively blocked the enhancing effects of glutamine or GABA on the CCK-stimulated pancreatic secretions. Because glutamine is a major precursor of GABA[18], the results strongly suggest that glutamine may enhance the CCK-stimulated pancreatic exocrine secretion by inducing secretion of GABA. So far, there is no direct evidence that GABA is secreted from the islet β-cells into the intra-pancreatic circulation although cultured β-cells secrete GABA into medium[14]. Thus, we examined whether GABA is secreted from the β-cells into the portal circulation of the pancreas in this study. When glutamine was added in the perfusate at concentrations of 1, 4 and 10 mmol/L, the GABA concentration in portal effluent was elevated dose-dependently. The effect of glutamine on the CCK-stimulated exocrine secretion was reduced in the pancreas isolated from rats treated with streptozotocin, a well-known β-cell toxin. Furthermore, not only at the basal state but also at the glutamine-infused state, the GABA concentration in portal effluent was much lower in the streptozotocin-treated pancreas than in the normal pancreas. The results firstly show that GABA is secreted by a secretagogue from islet β-cells into the intra-pancreatic circulation, which enhances the CCK-stimulated pancreatic exocrine secretion. There is good evidence that β-cells may secrete GABA. It has been reported that β-cells contain not only GABA but also GAD, a GABA synthesizing enzyme at high concentration[1,2,22]. β-cells include synaptic-like microvesicles that may be concerned with GABA secretion[22]. The pancreatic β-TC6 cells[13] or cultured rat β-cells[14] secrete GABA into the medium in response to glucose or glutamine. GABA may be also secreted from the GABAergic neurons in the pancreas because GABA-containing neuronal cell bodies locate at the periphery of islets, and numerous GABA-containing processes of the neurons extend into the exocrine pancreas[23]. Secretion of GABA from the GABA-containing neurons should be elucidated in future studies.

Endogenous GABA appears to exert the enhancing effects on the CCK-stimulated pancreatic exocrine secretion via $GABA_A$ receptors. In this study, the enhancing effects of glutamine on the CCK-stimulated pancreatic secretion were effectively blocked by bicuculline, a $GABA_A$ receptor antagonist[15,19]. Existence of $GABA_A$ receptors has been reported in pancreatic exocrine cells of neonatal rats[12] and in AR42J cells, a pancreatic cancer cell line[24]. Endogenous GABA may elevate the CCK-stimulated pancreatic exocrine secretion indirectly by modulating release of islet hormones. It has been reported that GABA or its agonist inhibits release of somatostatin[6,25], which inhibits CCK-stimulated pancreatic exocrine secretion[26,27]. We have already reported that a somatostatin antagonist further elevates the enhancing effects of GABA on CCK-stimulated pancreatic exocrine secretion[15]. It has been also documented that GABA stimulates release of insulin[8], which increases CCK-stimulated pancreatic exocrine secretion[28,29]. In addition, the GAD activity in the pancreatic islet is very low in mice suffering from insulin-dependent diabetes mellitus[9], in which pancreatic exocrine secretion is reduced[30]. Endogenous GABA may also enhance the intrinsic neuronal action on pancreatic exocrine secretion. Our previous studies showed that exogenous GABA further elevates pancreatic exocrine secretion evoked by electrical field stimulation as well as neurotransmitters such as gastrin-releasing peptide[27] and acetylcholine[32] in rat pancreas. Recently, GABA-sensitive neurons have been electrophysiologically observed in cat pancreas[33]. Although GABA could depolarize all ganglial

cells recorded in the study through $GABA_A$ receptor, only approximately 10% of neurons among them could generate action potential. Thus, it is unclear at present whether the GABA-induced neuronal activation results in modification of pancreatic exocrine secretion.

In summary, glutamine could further elevate CCK-stimulated pancreatic exocrine secretion of the isolated rat pancreas dose-dependently, which could be blocked by bicuculline. Glutamine could increase the GABA concentration in portal effluent of the isolated rat pancreas dose-dependently. The effects of glutamine on the CCK-stimulated pancreatic exocrine secretion as well as the GABA secretion were markedly reduced in the pancreas isolated from the streptozotocin-treated rat. Therefore, it is concluded that GABA can be secreted from islet β-cells into the islet-acinar portal system after administration of glutamine, which could enhance CCK-stimulated pancreatic exocrine secretion via $GABA_A$ receptors. The results strongly indicate that GABA in islet β-cells may be an islet hormone affecting exocrine secretion of the pancreas in rats.

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BASIC RESEARCH

Gastro-protective action of lafutidine mediated by capsaicin-sensitive afferent neurons without interaction with TRPV1 and involvement of endogenous prostaglandins

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Abstract

AIM: Lafutidine, a histamine H_2 receptor antagonist, exhibits gastro-protective action mediated by capsaicin-sensitive afferent neurons (CSN). We compared the effect between lafutidine and capsaicin, with respect to the interaction with endogenous prostaglandins (PG), nitric oxide (NO) and the afferent neurons, including transient receptor potential vanilloid subtype 1 (TRPV1).

METHODS: Male SD rats and C57BL/6 mice, both wild-type and prostacyclin IP receptor knockout animals, were used after 18 h of fasting. Gastric lesions were induced by the po administration of HCl/ethanol (60% in 150 mmol/L HCl) in a volume of 1 mL for rats or 0.3 mL for mice.

RESULTS: Both lafutidine and capsaicin (1-10 mg/kg, po) afforded dose-dependent protection against HCl/ethanol in rats and mice. The effects were attenuated by both the ablation of CSN and pretreatment with N^G-nitro-L-arginine methyl ester, yet only the effect of capsaicin was mitigated by prior administration of capsazepine, the TRPV1 antagonist, as well as indomethacin. Lafutidine protected the stomach against HCl/ethanol in IP receptor knockout mice, similar to wild-type animals, while capsaicin failed to afford protection in the animals lacking IP receptors. Neither of these agents affected the mucosal PGE_2 or 6-keto $PGF_{1\alpha}$ contents in rat stomachs. Capsaicin evoked an increase in $[Ca^{2+}]i$ in rat TRPV1-transfected HEK293 cells while lafutidine did not.

CONCLUSION: These results suggest that although both lafutidine and capsaicin exhibit gastro-protective action mediated by CSN, the mode of their effects differs regarding the dependency on endogenous PGs/IP receptors and TRPV1. It is assumed that lafutidine interacts with CSN at yet unidentified sites other than TRPV1.

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Key words: Lafutidine; A histamine H_2-receptor antagonist; Gastric protection; Prostaglandin; Capsaicin-sensitive afferent neuron; TRPV1

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INTRODUCTION

The mucosal integrity of the stomach is maintained by multiple factors, both paracrine and neuronal systems[1-4]. Prostaglandins (PGs)[1] and nitric oxide (NO)[4] are part of the paracrine system while capsaicin-sensitive afferent neurons play a central role in the neuronal protection of the stomach[2,3]. Studies have demonstrated that capsaicin, a selective stimulator of these afferent neurons, protects the gastric mucosa against various ulcerogenic stimuli such as necrotizing agents[2]. The protective action of capsaicin is mediated by these afferent neurons, because it is totally attenuated by chemical ablation of the neurons following pretreatment with a large dose of capsaicin[2,5]. Recently, the binding site of capsaicin has been identified and named transient receptor potential vaniloid subtype 1 (TRPV1)[6-8]. It is thought that capsaicin stimulates these afferent neurons through the activation of TRPV1, resulting in the release of the neurotransmitter calcitonin gene-related peptide (CGRP) and gastric protection[3].

Previous studies suggest that endogenous PGs sensitize sensory neurons to nociceptive stimulation[9,10]. Boku *et al*[11] recently reported a lack of release of CGRP in response to mild injury in the stomach of IP-receptor knockout mice. Ohishi *et al*[9] demonstrated using IP-receptor knockout mice that PGI_2 is a major nociceptive mediator in the acetic acid-induced writhing reaction. Since

capsaicin-induced gastric cytoprotection was attenuated by indomethacin and disappeared in IP-receptor knockout mice, it is assumed that endogenous prostacyclin (PGI_2) plays a supportive role in the mechanism of capsaicin-induced gastric cytoprotection, probably by sensitizing the sensory neurons through IP receptors[12].

Lafutidine, a histamine H_2-receptor antagonist [(±)-2-(furfurylsulfinyl)-N-[4-[4-(piperidinomethyl)-2-pyridyl]oxy-(Z)-2-butenyl]acetamide], is known to exhibit potent protective activity in the gastrointestinal mucosa, in addition to gastric anti-secretory action[13-15]. The protective action of lafutidine is independent of the anti-secretory effect and mediated by capsaicin-sensitive sensory neurons[14,15]. Indeed, the protective action of lafutidine is also abrogated by pretreatment with the CGRP antagonist $CGRP_{8-37}$ and the inhibitor of NO production N^G-nitro-L-arginine methyl ester (L-NAME), in addition to chemical ablation of capsaicin-sensitive afferent neurons[16]. However, it remains unknown whether this action of lafutidine results from interaction with TRPV1 or whether endogenous PGs are essential to the gastro-protective effect.

In the present study, we investigated the protective action of lafutidine with respect to the dependence on endogenous PGs and the interaction with TRPV1, and compared the results with those for capsaicin. In addition, we also examined the effect of lafutidine on HCl/ethanol-induced gastric lesions in mice lacking IP receptors, in an attempt to confirm the distinctive mechanism underlying the gastro-protection provided by lafutidine and capsaicin.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200-220 g; Nippon Charles River, Shizuoka, Japan) and C57BL/6 mice (25-30 g) were used. Mice lacking prostacyclin (PGI_2) IP-receptors were generated as described previously[11]. In brief, each gene encoding the IP receptors was individually disrupted, and chimeric mice were generated. These animals were then back crossed with C57BL/6 mice, and the resulting heterozygous litter mates [IP (+/-),] were bred to produce homozygous IP (-/-) mice. Homozygous mice were born at the predicted Mendelian frequency, grew normally, lived longer than 1 year and were fertile. The distribution of the IP receptor genes was verified by northern blot hybridization, which failed to detect messenger RNA encoding the respective receptors in IP (-/-) mice. The animals were deprived of food but allowed free access to tap water for 18 hr before the experiments. All studies were performed using 4-6 animals per group under unanesthetized conditions. The experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Induction of Gastric Lesions

The rats were given 1 mL of HCl/ethanol (60% in 150 mmo/L HCl) po through esophageal catheter, and killed 1 h later under deep ether anesthesia. The stomach was removed, inflated by injecting 10 mL of 1% formalin for 10 min to fix the inner wall, and opened along the greater curvature. The area (mm^2) of hemorrhagic lesions developed in the stomach was measured under a dissecting microscope with a square grid (×10). Lafutidine (1-10 mg/kg) or capsaicin (1-10 mg/kg) was given po 30 min before the administration of HCl/ethanol. In some cases, indomethacin (5 mg/kg, sc), L-NAME (20 mg/kg, sc) or capsazepine (TRPV1 antagonist: 12.5 mg/kg, po) was given 1 h or 30 min, respectively, before the administration of lafutidine or capsaicin. In addition, the protective effect of lafutidine and capsaicin against HCl/ethanol was also examined in rats with ablation of capsaicin-sensitive sensory neurons (chemical deafferentation). Chemical deafferentation was induced by s.c. injections of capsaicin once daily for three consecutive days (total dose: 100 mg/kg) 2 wk before the experiment[2, 5]. All capsaicin injections were performed under ether anesthesia, and the rats were pretreated with terbutaline (0.1 mg/kg, im) and aminophylline (10 mg/kg, im) to counteract the respiratory impairment associated with capsaicin injection. The effectiveness of the treatment was tested by examining the protective wiping movements of the eye.

In another experiment, wild-type mice and IP-receptor knockout mice were given HCl/ethanol po in a volume of 0.3 mL, and killed 1 hr later[17]. Then, the stomach was removed and treated with formalin, and the mucosa was examined for hemorrhagic lesions under a dissecting microscope, as described previously. In half the animals of each group, lafutidine (10 and 30 mg/kg) or capsaicin (10 mg/kg) was given po 30 min before the administration of HCl/ethanol.

Measurement of Mucosal PGE_2 and 6-keto $PGF_{1\alpha}$ Levels

Levels of PGE_2 in the rat gastric mucosa and those of 6-keto $PGF_{1\alpha}$ in the mouse stomach were measured 30 min after the po administration of lafutidine (10 mg) or capsaicin (10 mg/kg). In some cases, indomethacin (5 mg/kg) was given s.c. 1 h before the capsaicin. Under ether anesthesia, the stomachs were quickly removed, opened along the greater curvature, and rinsed with ice-cold saline. To separate the mucosal layer, the corpus mucosa was placed between two glass slides squeezed with a rubber band, and placed in hexane-frozen Dry Ice and acetone. These glasses were separated, and the mucosa was collected, weighed, and put in a tube containing 100% ethanol plus 0.1 mol/L indomethacin[18,19]. Then, the samples were homogenized, and centrifuged for 10 min at 12000 g at 4 ℃. The supernatant of each sample was used for the determination of PGE_2 and 6-keto $PGF_{1\alpha}$ levels by EIA with a PGE_2- and 6-keto $PGF_{1\alpha}$-kit, respectively (Cayman Chemical Co., Ann Arbor, MI).

Determination of the Ca^{2+} Response in VR1-Transfected HEK239 Cells

Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 15% fetal bovine serum (FBS, JRH Biosciences, KS) and 100 IU/mL of penicillin plus 100 μg/mL of streptomycin (Gibco BRL, MD) in an atmosphere of 5% CO_2 and 95% air at 37 ℃. HEK293 cells were seeded on glass cover slips coated with poly-L-lysine (Sigma Chem, MO) in 35 mm dishes. The cells grown to 70%-80% confluence were transfected with 3

μg of VR1-pCR3.1-Uni plasmid using LipofectA-MINE Reagent (Gibco BRL, MD) and were cultured in DMEM for 48 hr[20]. After the medium was changed once with calcium imaging buffer (CIB) consisting of 115 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 13.8 mmol/L D-glucose, and 20 mmol/L HEPES (adjusted to pH 7.4 with NaOH), cells were incubated with CIB containing 5 μmol/L Fura-2-AM (Dojin, Kumamoto, Japan) at room temperature for 45 min. Then, cells were washed twice with CIB. The mobilization of Ca^{2+} was monitored with an ARGUS-100/CA system (Hamamatsu Photonics, Hamamatsu, Japan). Dual images (340 and 380 nm excitation, 510 nm emission) were collected for 180 s. The $[Ca^{2+}]i$ was calculated from Fura-2 ratios by the equation described in a previous paper[20,21], and the maximal responses were determined. Capsaicin (10^{-8}-10^{-6} mol/L) or lafutidine (10^{-4} mol/L) was added to the medium.

Preparation of Drugs

Drugs used were lafutidine (UCB Japan, Tokyo, Japan), capsaicin (Nakarai Tesque, Kyoto, Japan), indomethacin, capsazepine, N^G-nitro-L-arginine methyl ester (L-NAME)(Sigma Chemicals, St. Louis, MO), terbutaline (Bricanyl^R, Fujisawa, Osaka, Japan) and aminophylline (Neophyllin^R, Eisai, Tokyo, Japan). Lafutidine was suspended with 0.5% carboxymethylcellulose solution (CMC: Nacalai Tesque). Capsaicin was dissolved in a Tween 80-ethanol solution (10% ethanol, 10% Tween and 80% saline, w/w: Wako, Osaka, Japan) for sc injection while it was suspended in a 0.5% CMC for topical application. Indomethacin was suspended in saline with a drop of Tween 80 (Wako, Osaka, Japan). Other agents were dissolved in saline. Each agent was prepared immediately before use and given in a volume of 0.5 mL per 100 g body weight (rat) for po or sc administration or 0.1 mL per 10 g body weight (mouse) for po administration. Control animals received saline instead of the active agent.

Statistical analysis

Data are presented as the mean ± SE for 4-6 animals per group. Statistical analyses were performed using a two-tailed Dunnett's multiple comparison test, and values of $P < 0.05$ were regarded as significant.

RESULTS

Effect of Lafutidine and Capsaicin on HCl/Ethanol-Induced Gastric Lesions in Rats

Oral administration of HCl/ethanol (60% in 150 mmol/L HCl) produced multiple lesions in the glandular mucosa, along the long axis of the rat stomach. These lesions were dose-dependently prevented by prior administration of lafutidine (1-10 mg/kg, po). A significant effect was observed even at 1 mg/kg and the inhibition was 36.8% (Figure 1A). Likewise, pretreatment of the animals with capsaicin (1-10 mg/kg, po) also provided dose-dependent protection against HCl/ethanol-induced gastric lesions and a significant effect was observed at 3 mg/kg or greater (Figure 1B). The ED_{50} of lafutidine and capsaicin was 1.4 mg/kg and 3.1 mg/kg, respectively. The protective effects of both lafutidine (10 mg/kg) and capsaicin (10 mg/kg)

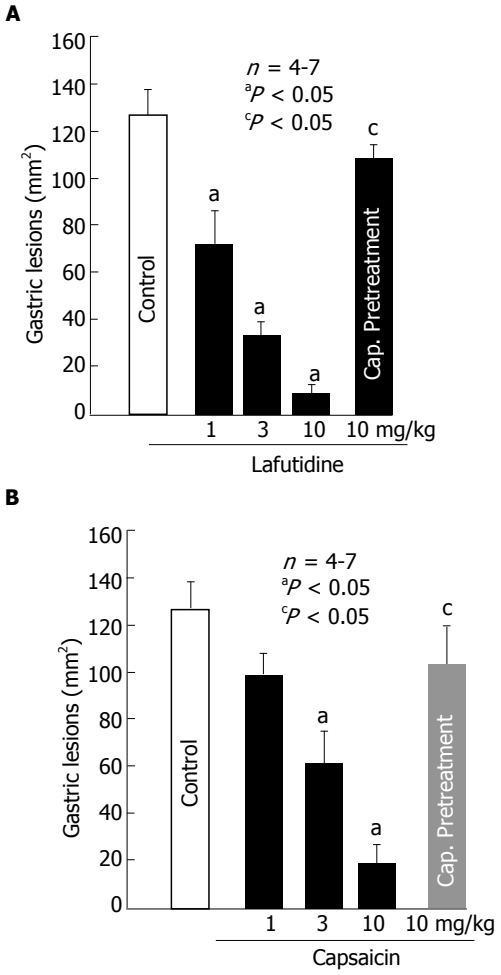


Figure 1 Effects of lafutidine (**A**) and capsaicin (**B**) on HCl/ethanol-induced gastric lesions in rats. The animals were given 1 mL of HCl/ethanol (60% ethanol in 150 mmol/L HCl) po and killed 1 h later. Lafutidine (1-10 mg/kg) and capsaicin (1-10 mg/kg) were administered po 30 min before HCl/ethanol. Ablation of capsaicin-sensitive afferent neurons was achieved by consecutive s.c. injections of capsaicin (total dose of 100 mg/kg) once daily for 3 d 2 wk before the experiment. Data are presented as the mean ± SE from 4-7 rats. $^aP < 0.05$, $^cP < 0.05$ *vs* control.

were almost totally reversed by chemical ablation of capsaicin-sensitive afferent neurons following capsaicin pretreatment (Figures 1 A and 1B). Sensory deafferentation by itself did not significantly affect the development of gastric lesions in response to HCl/ethanol (data not shown).

Effect of Capsazepine, L-NAME and Indomethacin on the Gastroprotection Afforded by Lafutidine and Capsaicin

Oral administration of lafutidine or capsaicin at 10 mg/kg provided a marked protection against HCl/ethanol-induced gastric lesions in rats. The protective effect of capsaicin was significantly negated by pretreatment with capsazepine (12.5 mg/kg, po), and the degree of protection afforded by capsaicin was 19.7%, which is significantly less than that (85.4%) observed in the vehicle-treated normal rats (Figure 2). Likewise, pretreatment of the animals with indomethacin (5 mg/kg, sc) also significantly abrogated the protective action of capsaicin (Figure 3). Although capsaicin afforded significant protection against HCl/ethanol-induced gastric lesions, even in the presence

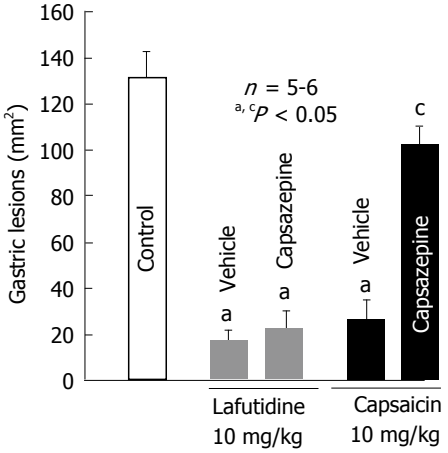


Figure 2 Effect of capsazepine on the protective action of lafutidine or capsaicin against HCl/ethanol-induced gastric lesions in rats. The animals were given 1 mL of HCl/ethanol (60% ethanol in 150 mmol/L HCl) po and killed 1 h later. Lafutidine or capsaicin was given po 30 min before HCl/ethanol. Capsazepine (12.5 mg/kg) was administered po 30 min before lafutidine or capsaicin. Data are presented as the mean ± SE from 4-7 rats. $^aP < 0.05$ *vs* control, $^cP < 0.05$ *vs* vehicle.

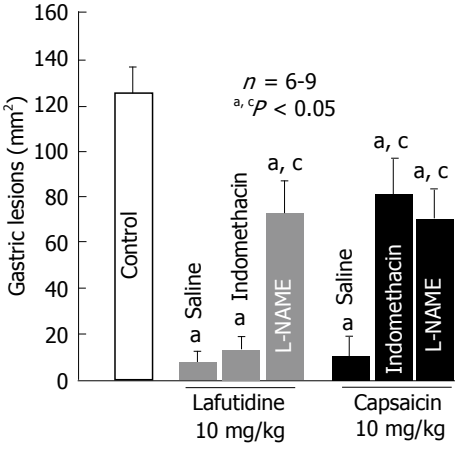


Figure 3 Effects of indomethacin and L-NAME on the protective action of lafutidine or capsaicin against HCl/ethanol-induced gastric lesions in rats. The animals were given 1 mL of HCl/ ethanol (60% ethanol in 150 mmol/L HCl) po and killed 1 h later. Lafutidine (10 mg/kg) or capsaicin (10 mg/kg) was given po 30 min before HCl/ethanol. Indomethacin (5 mg/kg) or L-NAME (20 mg/kg) was administered sc 1 h before HCl/ethanol. Data are presented as the mean ± SE from 6-9 rats. $^aP < 0.05$ *vs* control, $^cP < 0.05$ *vs* saline.

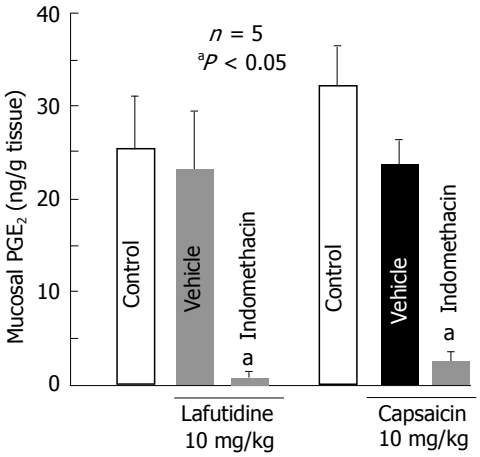


Figure 4 Effects of lafutidine and capsaicin on mucosal PGE_2 contents in rat stomachs. The animals were given lafutidine (10 mg/kg) or capsaicin (10 mg/kg) po and killed 1 h later. Indomethacin (5 mg/kg) was administered sc 30 min before capsaicin or lafutidine. Gastric mucosal PGE_2 content was determined by EIA. Data are presented as the mean ± SE from 5 rats. $^aP < 0.05$ *vs* control.

of indomethacin, the degree of protection was decreased to 31.9%, which is much less than that (89.7%) observed in the saline control group. By contrast, neither capsazepine nor indomethacin significantly affected the protective action of lafutidine against HCl/ethanol-induced gastric lesions (Figures 2 and 3). Lafutidine had a potent protective effect in the absence or presence of indomethacin and the degree of protection was 91.6% or 85.7%, respectively. On the other hand, the protective action of capsaicin as well as lafutidine was curtailed by prior administration of L-NAME (20 mg/kg, sc), the inhibitor of NO production. The degree of protection was 41.7% and 38.7%, respectively, and both values were significantly less than those observed in the absence of L-NAME.

Effect of Lafutidine and Capsaicin on PGE_2 Content in Rat Stomachs

Oral administration of capsaicin (10 mg/kg) did not significantly affect the mucosal PGE_2 content determined at 0.5 h after the administration (Figure 4). Likewise, the mucosal PGE_2 content was not significantly altered by the po administration of lafutidine at 10 mg/kg. Prior administration of indomethacin (5 mg/kg, sc) markedly reduced the PGE_2 content in the presence of capsaicin or lafutidine and the inhibition was 84.7% or 94.2%, respectively.

Effect of Lafutidine and Capsaicin on HCl/Ethanol-Induced Gastric Lesions in Mice

It was found that lafutidine, unlike capsaicin, provided gastric protection against HCl/ethanol-induced damage in rats, even in the presence of indomethacin. In addition, we previously found that endogenous PGs modulate the protective action of capsaicin through the activation of IP receptors[12]. To further investigate the involvement of IP receptors in the gastro-protection afforded by lafutidine, we examined the effect of this agent on ethanol-induced gastric lesions in both wild-type and IP receptor knockout mice, in comparison with that of capsaicin.

Intragastric administration of HCl/ethanol (0.3 mL) caused hemorrhagic lesions in the stomach of wild-type mice and the lesion area was 34.1 ± 4.9 mm² (Figure 5). HCl/ethanol produced gastric lesions in IP-receptor knockout animals, similar to wild-type mice, and the severity of these lesions did not differ between two groups. Similar to the findings in rats, the severity of these lesions in wild-type mice was significantly reduced by prior administration of capsaicin (10 mg/kg, po) and the inhibition was 82.3%. Likewise, intragastric lafutidine (3 and 10 mg/kg) significantly reduced the severity of these lesions in wild-type animals in a dose-dependent manner and the degree of protection was 68.2% and 79.8%, respectively.

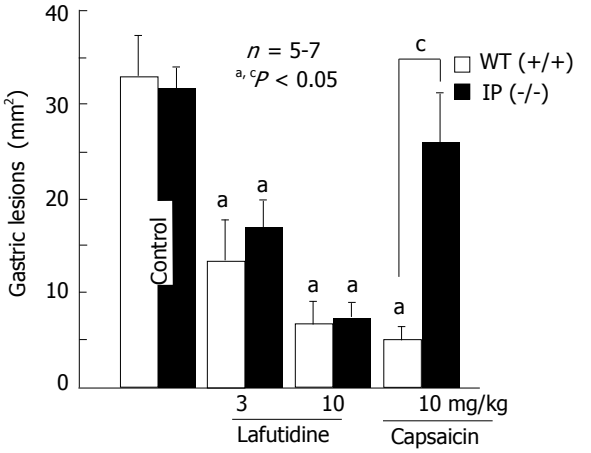


Figure 5 Effects of lafutidine and capsaicin on HCl/ethanol-induced gastric lesions in wild-type and IP-receptor knockout mice. The animals were given 1 mL of HCl/ethanol (60% ethanol in 150 mmol/L HCl) po and killed 1 h later. Lafutidine (10-30 mg/kg) or capsaicin (10 mg/kg) was given po 30 min before HCl/ethanol. Data are presented as the mean ± SE from 5-7 rats. $^aP < 0.05$ *vs* control.

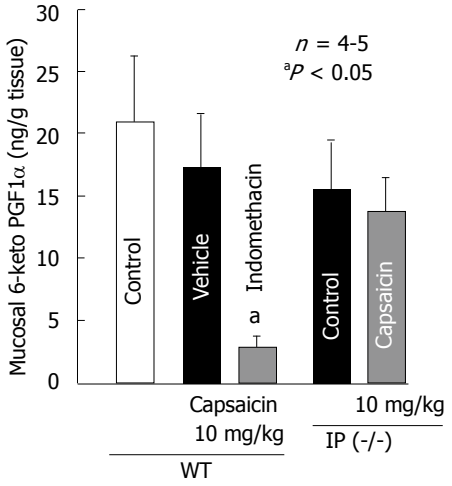


Figure 6 Effects of lafutidine and capsaicin on gastric mucosal 6-keto-$PGF_{1\alpha}$ levels in mice. The animals were given capsaicin (10 mg/kg) po and killed 1 h later. Indomethacin (5 mg/kg) was administered sc 30 min before capsaicin. Gastric mucosal 6-keto-$PGF_{1\alpha}$ levels were determined by EIA. Data are presented as the mean ± SE from 4-5 rats. $^aP < 0.05$ *vs* control.

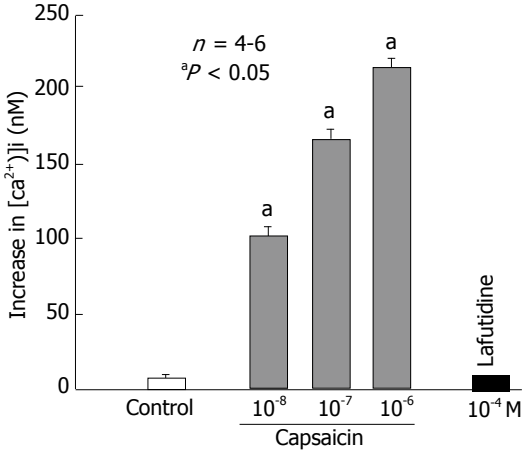


Figure 7 Changes in the intracellular concentration of Ca^{2+} in response to capsaicin and lafutidine in HEK293 cells transfected with VR1. Rat VR1 was introduced into HEK293 cells. Capsaicin (10^{-8}-10^{-6} mol/L) or lafutidine (10^{-4} mol/L) was added to the medium. Data are presented as the mean ± SE from 3 experiments. $^aP < 0.05$ *vs* control.

This agent protected the stomach of IP receptor knockout mice against HCl/ethanol as effective as observed in wild-type animals. However, the capsaicin did not afford significant protection in the animals lacking IP receptors and the degree of protection was 21.4%, which is significantly less than that (82.3%) observed in wild-type mice.

Effect of Capsaicin on 6-keto $PGF_{1\alpha}$ Content in Mouse Stomach

Since the protective action of capsaicin was not observed in IP-receptor knockout mice, we measured the effect of capsaicin on gastric 6-keto $PGF_{1\alpha}$ production in both wild-type and IP-knockout mice. Oral administration of capsaicin (10 mg/kg) did not significantly affect 6-keto $PGF_{1\alpha}$ content, same as the effect on PGE_2 content in rat stomachs (Figure 6). Indomethacin (5 mg/kg, sc) markedly decreased 6-keto $PGF_{1\alpha}$ content in the presence of capsaicin and the reduction was 84.1%. Similarly, capsaicin had no effect on 6-keto $PGF_{1\alpha}$ production in IP-receptor knockout mice.

Effect of Lafutidine and Capsaicin on the Ca^{2+} Concentration in Rat VR1-Transfected HEK239 Cells

To investigate the interaction of lafutidine with VR1 receptors, we examined the effect of lafutidine on the concentration of Ca^{2+} in rat VR1-transfected HEK239 cells. Addition of capsaicin (10^{-8}-10^{-6} mol/L) evoked a dose-dependent and significant increase in $[Ca^{2+}]i$ in the HEK239 cells (Figure 7). By contrast, lafutidine even at 10^{-4} mol/L did not effect the $[Ca^{2+}]i$.

DISCUSSION

Lafutidine, a histamine H_2 receptor antagonist, has been shown to afford protection in the gastrointestinal tract, partly through capsaicin-sensitive afferent neurons[16]. However, the precise mechanism of this action, including the dependence on endogenous PGs and the interaction with TRPV1, remains unknown. The present study confirmed that lafutidine protected the stomach from HCl/ethanol-induced injury through the activation of capsaicin-sensitive afferent neurons, similar to capsaicin. furthermore, we demonstrated that the action mode of these two agents differs in terms of dependency on PGs and interaction with TRPV1. It is assumed that lafutidine interacts with capsaicin-sensitive afferent neurons at yet unidentified sites other than TRPV1.

Lafutidine, an antiulcer drug developed in Japan[13], has been shown to have a potent antisecretory effect due to a blockage of histamine H_2 receptors and mucosal protective action in the gastrointestinal tract including the esophagus, stomach, small intestine and large intestine[14-16,22,23]. The latter effect is independent of the antisecretory action and mediated by capsaicin-sensitive afferent neurons[14,15,22,23], because the effect was not mimicked by cimetidine, another H_2-receptor antagonist, and was significantly abro-

gated by chemical ablation of the afferent neurons. In the present study, we also confirmed that the development of gastric lesions in response to HCl/ethanol was dose-dependently prevented by prior administration of lafutidine, and this effect was totally attenuated by sensory defunctionalization following pretreatment with a large dose of capsaicin. It is known that stimulation of capsaicin-sensitive afferent neurons releases calcitonin gene-related peptide (CGRP), and the action of CGRP is in part mediated by endogenous nitric oxide (NO)[3]. Indeed, several studies showed that the gastro-protective action of capsaicin, a selective stimulant of these afferent neurons, is attenuated by the NO synthase inhibitor L-NAME as well as the CGRP antagonist $CGRP_{8-37}$[16,24]. Onodera *et al*[16] reported that the protective effect of lafutidine on ethanol-induced gastric lesions was also significantly mitigated by prior administration of CGRP or L-NAME, suggesting the involvement of both CGRP and NO in the protective action. We previously reported that the protective effect of lafutidine on indomethacin-induced small intestinal lesions was abrogated by pretreatment with L-NAME as well as sensory deafferentation[15,25]. The present study also showed that pretreatment of the animals with L-NAME significantly attenuated the protective action of both capsaicin and lafutidine against HCl/ethanol-induced gastric lesions.

Interestingly, the presence of endogenous PGs is required for the expression of various capsaicin-induced actions in the stomach, including the mucosal protection, the increase in mucosal blood flow, and the stimulation of bicarbonate secretion[5,26,27]. Indeed, the gastro-protective effect of capsaicin was curtailed when animals were pretreated with indomethacin to inhibit PG production[5,28]. We confirmed in this study that the protective effect of capsaicin on HCl/ethanol-induced gastric lesions was significantly mitigated in the presence of indomethacin, confirming the involvement of endogenous PGs in this action. Consistent with our previous study[12], we observed that capsaicin failed to exhibit gastro-protective action in prostacyclin IP receptor knockout mice, suggesting a modulator role for IP receptors in the facilitation by endogenous PGs of gastric protection mediated by capsaicin-sensitive afferent neurons. Recently, it has been shown that endogenous phosphatidylinositol-4, 5-bisphosphate ($PtdIns(4,5)P_2$) inhibits TRPV1, and the repression can be alleviated by agents that activate phospholipase C (PLC)[29,30]. Bradykinin potentiates the activation of TRPV1 by capsaicin through the hydrolysis of $PtdIns(4,5)P_2$ in a PLC-dependent manner[30]. Thus, PGs might sensitize the afferent neurons to capsaicin through IP receptors, by somehow releasing TRPV1 from $PtdIns(4,5)P_2$-mediated inhibition. However, as shown in the present study, lafutidine exhibited a gastro-protective action against HCl/ethanol-induced damage in indomethacin-pretreated rats as well as in IP receptor knockout mice, as effective as in the respective control animals, strongly suggesting that the effect is independent of endogenous PGs. It is known that mucosal acidification increases HCO_3^- secretion in the stomach, and this response was attenuated by indomethacin [31]. We previously reported that lafutidine restored the response to capsaicin even in the presence of indomethacin [21]. It is thus assumed that lafutidine somehow plays a supportive role in the mechanism of action mediated by capsaicin-sensitive afferent neurons, similar to endogenous PGs.

The peptidergic afferent innervation of the stomach has been demonstrated in various species of animals including the rat [32]. Many studies including ours have shown that lafutidine affords gastric protection through capsaicin-sensitive sensory neurons, similar to capsaicin. However, it remains unknown how lafutidine interacts with these neurons. It is known that capsaicin stimulates the afferent neurons through the activation of TRPV1, resulting in the liberation of CGRP, the predominant neurotransmitter of spinal afferents in the rat stomach, and by so doing exerts a gastro-protective action [33]. This idea was supported by the present finding that the protective effect of capsaicin was totally inhibited by capsazepine, an antagonist of TRPV1. It is possible that lafutidine also interacts with TRPV1 to facilitate the activity of afferent neurons, similar to capsaicin. Nishihara *et al*[34], however, reported that lafutidine alone did not cause a release of CGRP from the stomach but enhanced the release induced by a submaximal dose of capsaicin. They also showed that lafutidine had no effect on the specific binding of [^{3}H]-resiniferatoxin to TRPV1[34]. These results suggest that although lafutidine modulates the activity of capsaicin-sensitive afferent nerves, this effect is not brought about through interaction with TRPV1. We also observed that lafutidine had no effect by itself on $[Ca^{2+}]i$ in rat VR1-transfected HEK 239 cells, although capsaicin produced a dose-dependent increase in $[Ca^{2+}]i$ in these cells. Thus, it is unlikely that lafutidine modulates the activity of capsaicin-sensitive afferent neurons through interaction with TRPV1. As observed in the present study, the TRPV1 antagonist capsazepine did not significantly influence the protective effect of lafutidine, confirming that this agent does not interact with TRPV1.

As mentioned above, it was concluded that although lafutidine exhibits gastroprotective action mediated by capsaicin-sensitive neurons, similar to capsaicin, the mode of its effect differs regarding the dependency on PGs/IP receptors or TRPV1. It is assumed that lafutidine interacts with capsaicin-sensitive afferent neurons at unidentified sites other than TRPV1 and mimics the effect of endogenous PGs to modulate the activity of the afferent neurons. The exact mechanism by which lafutidine interacts with capsaicin-sensitive afferent neurons awaits further study.

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BASIC RESEARCH

Helicobacter species ribosomal DNA in the pancreas, stomach and duodenum of pancreatic cancer patients

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Abstract

AIM: To determine whether gastric and enteric *Helicobacter* species are associated with pancreatic cancer.

METHODS: Patients with exocrine pancreatic cancer (n = 40), neuroendocrine cancer (n = 14), multiple endocrine neoplasia type 1 (n = 8), and chronic pancreatitis (n = 5) were studied. Other benign pancreatic diseases (n = 10) and specimens of normal pancreas (n = 7) were included as controls. Pancreatic tissue specimens were analyzed by *Helicobacter*-specific PCR-assay and products were characterized by denaturing gradient electrophoresis and DNA-sequencing. From a subset of the pancreatic cancer patients, gastric and/or duodenal tissue as well as gallbladder and ductus choledochus tissue were analyzed. Gallbladder and choledochus samples were included as controls. Stomach and duodenum samples were investigated to analyze whether a gastric *helicobacter* might disseminate to the pancreas in pancreatic cancer patients. Pancreatic specimens were analyzed by *Bacteroides*-specific PCR for detecting the translocation of indigenous gut microbes to the diseased pancreas.

RESULTS: *Helicobacter* DNA was detected in pancreas (tumor and/or surrounding tissue) of 75% of patients with exocrine cancer, 57% of patients with neuroendocrine cancer, 38% of patients with multiple endocrine neoplasia, and 60% of patients with chronic pancreatitis. All samples from other benign pancreatic diseases and normal pancreas were negative. Thirty-three percent of the patients were *helicobacter*-positive in gastroduodenal specimens. Surprisingly, *H. bilis* was identified in 60% of the positive gastroduodenal samples. All gallbladder and ductus choledochus specimens were negative for *helicobacter*. *Bacteroides* PCR-assay was negative for all pancreatic samples.

CONCLUSION: *Helicobacter* DNA commonly detected in pancreatic cancer suggests a possible role of the emerging pathogens in the development of chronic pancreatitis and pancreatic cancer.

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Key words: Pancreatic cancer; *Helicobacter* species; Polymerase chain reaction; DNA-sequence analysis

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INTRODUCTION

Pancreatic carcinoma, an extremely aggressive cancer with very poor prognosis, is one of the leading causes of cancer-related death in the Western world[1]. Consistently reported risk factors are age and cigarette smoking[1], whereas approximately 5% of pancreatic cancers seem to be primarily related to genetic traits[2]. Some early case control and data register studies suggested that chronic pancreatitis is a risk factor for pancreatic cancer[3, 4], which has been confirmed recently in prospective studies of chronic pancreatitis[5, 6]. *Helicobacter pylori (H pylori)*, the prototype species of the genus *Helicobacter*, which colonizes the stomach mucosa and causes acute and chronic gastritis as well as peptic ulcer disease, is a major predisposing factor for gastric cancer in human beings[7]. Other *Helicobacter* and *Campylobacter* species, including bile tolerant enteric *Helicobacter* species, colonize the intestine and hepatobiliary tract of many mammals and birds. *Helicobacter hepaticus* is the type organism of the enteric *Helicobacter* species and induces chronic active hepatitis, liver fibrosis, hepatocellular carcinoma, as well as inflammatory bowel disease in susceptible inbred strains of mice[8, 9]. Enteric *Helicobacter* species such as *H. pullorum*, *H. canis* and *H. cinaedi*, are associated with hepatitis in poultry, dogs, and macaques,

respectively, as well as gastroenteritis and bacteremia in human beings[9]. *H pylori* and the enteric species *H. bilis*, as well as *H pylori*-like *H.* species 'liver', are associated with biliary tract cancer, some chronic liver diseases as well as hepatocellular carcinoma in humans[10-14]. Two serology-based case-control studies have shown an association between *H pylori* and pancreatic cancer[15, 16], suggesting a possible relationship between *helicobacter* infections and pancreatic cancer development, which is supported by a recent study of *Helicobacter* species in a small number of patients with pancreatic exocrine cancer[17].

The purpose of the present investigation was to analyze the prevalence of gastric and enteric *Helicobacter* species DNA in samples from pancreatic cancer and chronic pancreatitis. Specimens from some benign pancreatic diseases (cysts or adenoma) as well as normal pancreatic tissue from patients with colon or choledochus cancer were included as controls. Translocation of some enteric bacteria to a diseased pancreas was analyzed by *Bacteroides* genus-specific PCR assay on pancreatic tissue.

MATERIALS AND METHODS

Patients

All patients were operated on at the Department of Surgery, Lund University Hospital. Formalin-fixed paraffin-embedded pancreatic tissue samples from 84 patients were obtained from the Department of Pathology at the same hospital. Prior to deembedding, one pathologist (U.S) reviewed all samples and approximately 100 mg of each tissue type was taken from the paraffin blocks with the tip of a scalpel. By carefully comparing the blocks with the slides, it was ascertained that a pure tissue type, i.e. tumor or normal etc, was obtained.

The tissue samples were from consecutive patients with primary exocrine cancer, predominantly of ductal type (PC) (n = 40, 20 females, mean age 59 years, range 44-77 years), neuroendocrine cancer (NE) (n = 14, 6 females, mean age 58 years, range 15-84 years), and multiple endocrine neoplasia type 1 (MEN) (n = 8, 1 female, mean age 52 years, range 42-69 years). In addition to a tumor specimen available from all 62 PC-, NE- and MEN patients, a sample of adjacent normal tissue was obtained from 41 patients (66%). Atrophic pancreatic tissue was available from 13 of the PC patients. Thus, 116 pancreatic tissue samples (1.9 samples per patient in average) were analyzed. Samples from patients with chronic pancreatitis (CP) of alcoholic, idiopathic, and epithelioid cell granulomatosis etiology (n = 5, 1 female, mean age 52 years, range 42-79 years), were also examined.

Pancreatic tissue specimens from patients with benign (other than pancreatitis) pancreatic diseases (n = *10*, 8 females, mean age 55 years, range 24-71 years) such as mucinous cystadenoma (n = 5), serous cystadenoma (n = 3), pancreatic cysts (n = 2), as well as histologically normal pancreatic tissue samples from patients with cancer of ductus choledochus (n = 4, 1 female, mean age 65 years, range 55-75 years), colon (male, mean age 60 years), duodenum (male, mean age 64 years), and retroperitoneal fibrosis (male, mean age 57 years), were included as controls (collectively denoted C). Gastric tissue samples of the antrum and/or fundus, as well as duodenal samples, were obtained from 23 PC patients and four NE- and MEN patients. An average of 2.5 stomach and/or duodenum specimens was tested per patient. Specimens of the gallbladder (n = 18) and ductus choledochus (n = 8) were also obtained from PC-, NE- and MEN patients. This study was approved by the Research Ethics Committee at Lund University (LU 726-02).

Bacterial strains

Bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Culture Collection of the University of Gothenburg (CCUG, Gothenburg, Sweden). Genomic DNA of *Bacteroides fragilis* (ATCC 25285), *H pylori* (CCUG 17874), *H. bilis* (CCUG 38995), *H. pullorum* (CCUG 33840), *H.* sp. flexispira (CCUG 23435), *H. hepaticus* (CCUG 33637), *Escherichia coli* (ATCC 25922), *Campylobacter jejuni* (ATCC 33560), *Clostridium difficile* (ATCC 9689), *Proteus mirabilis* (ATCC 43071), *Klebsiella pneumoniae* (ATCC 10031), *Enterococcus faecalis* (ATCC 29212) and *Enterobacter cloacae* (CCUG 6323) was extracted from 10^7 bacterial cells by the QIAamp® DNA Mini Kit (Qiagen) as described below.

Preparation of DNA

Paraffin-embedded tissue samples were heated at 60 ℃ for 10 min to melt excess paraffin, aseptically transferred to new micro-centrifuge tubes, washed in xylene for 2 × 5 min, rehydrated through graded ethanol (990 mL/L and 950 mL/L for 2 × 5 min and 700 mL/L for 5 min), and finally washed for 5 min in double-distilled water. Subsequently, the samples were homogenized in 170 mmol/L phosphate-buffered saline pH 7.2 by using a plastic micro-centrifuge tube-adapted pestle. Pancreatic tissue samples were homogenized at 10 g/L and other gastrointestinal specimens at 40-50 g/L. DNA was extracted from 100 μL of each homogenate by the QIAamp® DNA Mini Kit Tissue protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All DNA samples were stored at -20 ℃.

Helicobacter genus-specific PCR

DNA-extracts were amplified in a GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, US) by semi-nested PCR-assay for *Helicobacter* species as previously described[18] with primers constructed by Goto *et al*[19]. The forward primer 1F (5´-CTATGACGGGTATCCGGC-3´) and reverse primer 1R (5´-CTCACGACACGAGCTGAC-3´) were used in the first step. In the second step, primer 1F and reverse primer 2R (5´-TCGCCTTCGCAATGAGTATT-3´) were used. Precautions were taken to minimize the risk of PCR cross contamination as described recently[20]. Detection of PCR-products was done in agarose gels as described previously[18].

Bacteroides genus-specific PCR

To rule out the non-specific translocation of a common gut microbe to the pancreas among the studied patients, a pancreatic tissue sample (n = 84) from each patient was amplified

by nested PCR with primers for the glutamine synthase gene of *Bacteroides* species. Primers BFR-1 (5´-ACTCTTTGTATC CCGACGATT-3´) and BFR-2 (5´-GAGGTTGATGCCT-GTATCGGT-3´), described by Kane *et al*[21], were used. For the second step, internal primers (forward primer BFR-3: 5´-GACAAAAACATCACCCGGGT-3´ and reverse primer BFR-4: 5´-GCCCAGCTTGTGACACTCTATT-3´), based on the sequence of the BFR1-BFR2 PCR-product, were constructed using the Vector NTI® Suite version 8.0 (Informax, Frederick, MD, US). The specificity of the *Bacteroides* species PCR-assay was evaluated using strains from the ATCC and CCUG. PCR-mixtures were prepared as described previously[18]. Amplification conditions for the first step were at 94 ℃ for 4 min; 30 cycles at 94 ℃ for 30 s, at 60 ℃ for 30 s, at 72 ℃ for 45 s and finally at 72 ℃ for 5 min. Conditions for the second step were at 94 ℃ for 10 min; 35 cycles at 94 ℃ for 30 s, at 60 ℃ for 30 s, at 72 ℃ for 30 s and finally at 72 ℃ for 5 min. Genomic DNA (0.1 ng) of *B fragilis* was used as a positive control.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) analysis of the V6-7 region of *Helicobacter* species 16S rDNA was performed as described previously[18]. Diluted (10×) first step PCR-products were amplified in the second step using forward primer GC-1F (5´-GCGGCCGCCCGTCCC-GCCGCCCCCGCCCCGCCGCGGCCGCCTAT-GACGGGTATCCGGC-3´) and 2R as described above. Electrophoresis was carried out using a *DCode*™ electrophoresis unit (BioRad, Hercules, CA, US). A mixture of amplicons of type strain DNA of *H pylori* (CCUG 17874), *H. bilis* (CCUG 38995), *H. pullorum* (CCUG 33840), *H.* sp. flexispira (CCUG 23435), and *H. hepaticus* (CCUG 33637) was used as gel migration markers.

DNA-sequence analysis

Nucleic acid products of the *Helicobacter* genus-specific PCR-assay were purified from agarose gels using the Montage DNA Gel Extraction Kit (Millipore, Bedford, MA, US), or from DGGE-gels as described previously[18]. DNA-sequence reactions were performed using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems) with modifications. One microliter of a BigDye™ mix and 1.5 μL of sequencing buffer (10 μL of 10× PCR-buffer II, 6 μL 25 mmol/L $MgCl_2$, 4 μL double-distilled water) were prepared in a total volume of 10 μL with primers (1F or 2R) and template according to the manufacturer's instructions. Products of the sequence reaction were aligned and the closest homologous DNA was identified by BLASTn-analysis as described elsewhere[17].

RESULTS

Helicobacter PCR of pancreas

As a rule, two pancreatic specimens were obtained from each patient in the PC-, NE- and MEN-groups. If at least one specimen was positive, the patient was considered *Helicobacter*-positive. Hence, 75% (tumor and/or surrounding tissue) of the PC-, 57% of the NE-, and 38% of the MEN patients were positive for the genus *Helicobacter* (Table 1). Three (one alcoholic, one idiopathic and one with epithelioid cell granulomatosis) of five patients with chronic pancreatitis were *Helicobacter*-positive. All benign tissue samples from cystadenoma- and pancreas cyst patients, as well as pancreatic tissue from the remaining C-patients, were negative for the genus *Helicobacter* (Table 1).

Table 1 ***Helicobacter*** **genus-specific 16S ribosomal DNA PCR-assay (positive/total)**

Patient group	*n*	Pancreas	Stomach/ duodenum	Gallbladder	Ductus choledochus
PC	40	30/40	7/23	0/12	0/6
NE	14	8/14	1/2	0/4	0/1
MEN	8	3/8	1/2	0/2	0/1
CP	5	3/5	_	–	–
C	17	0/17	–	–	–

PC: pancreatic exocrine cancer; NE: neuroendocrine cancer; MEN: multiple endocrine neoplasia type 1; CP: chronic pancreatitis; C: cysts, cystadenoma and remaining controls.

Table 2 ***Helicobacter*** **genus-specific PCR in tumors and surrounding tissue**

Patient group	Tumor (*n* = 62)	Atrophic (*n* = 13)		Normal (*n* = 41)	
		+	–	+	–
PC	+ (*n* = 19)	4	3	1	10
	– (*n* = 21)	5	1	7	4
NE	+ (*n* = 8)	–	–	1	5
	– (*n* = 6)	–	–	0	5
MEN	+ (*n* = 3)	–	–	0	3
	– (*n* = 5)	–	–	0	5

PC: pancreatic exocrine cancer; NE: neuroendocrine cancer; MEN: multiple endocrine neoplasia type 1.

Helicobacter DNA was detected in 48% of tumors of PC patients (11 PC patients were PCR-negative in the tumor but positive in surrounding normal or atrophic tissue, hence, a higher number of PC patients [75%] compared with that of PC tumor patients [48%] were *Helicobacter*-positive), 57% of neuroendocrine tumors, and 38% of MEN tumors (Table 2). Atrophic and normal pancreatic tissues of the PC patients were positive in 69% and 36% of the samples, respectively, whereas only 5% of normal pancreatic tissues surrounding NE and MEN tumors were *helicobacter*-positive (Table 2). Commonly, if a PC patient was positive for *Helicobacter* species in a tumor sample, normal tissue was often negative and vice versa. Positive atrophic tissue was common in both tumor-negative and positive samples (Table 2).

Helicobacter PCR in the stomach, duodenum, gallbladder and ductus choledochus

Stomach and/or duodenum samples demonstrated a *Helicobacter*-positive result in 33% of the patients (Table 1). Of the 40 PC patients, stomach and/or duodenum was obtained from 23. Among these 23 patients, 30% were positive in the stomach and/or duodenum whereas 74% were positive in the pancreas. Four PC patients and one

Table 3 Distribution of *Helicobacter* 16S ribosomal DNA sequences identified using DNA-sequence and/or DGGE-analysis of the V6-region

Helicobacter species	Pancreatic tissue				Gastric or duodenal samples
	Normal[1]	Atrophic[2]	Tumor[3]	CP[4]	
H pylori	7	6	12	2	2
H bilis					6
H hepaticus				2	
H sp. flexispira			7		
H cineadi			2		

[1] Tissue adjacent to tumor; [2] Atrophic tissue (PC only); or [3] Tumor from PC-, NE- and MEN patients; [4]Chronic pancreatitis.

NE patient were simultaneously positive both in the pancreas and in a gastric and/or duodenal specimen (Figure 1). All gallbladder as well as ductus choledochus tissue samples were negative in the *Helicobacter* genus-specific PCR (Table 1).

Bacteroides genus-specific PCR

The expected 600-bp product was amplified with genomic DNA of *B. fragilis* as a template in the first step of the assay. However, after nested analysis, the expected fragment of 228-bp was also produced with *E. cloacae* DNA. Extracted genomic DNA of the other tested reference strains was negative. A pancreatic sample from each patient (n = 84), including tumor-, benign-, and normal tissue, was analyzed. None of the samples showed positive amplification for the genus *Bacteroides* in the nested PCR-assay.

DGGE- and DNA sequence analysis

Forty-six *Helicobacter* 16S ribosomal DNA PCR-products were identified by DGGE, and 20 of those were subjected to DNA-sequence analysis. DGGE revealed four migration profiles, similar to the reference strains of *H pylori*, *H.* sp. flexispira, *H. bilis* and *H. hepaticus* (Figure 1). The two methods were in concordance except for two PCR-products migrating with *H.* sp. flexispira in DGGE analysis but with the highest similarity to *H. cinaedi* after sequence- and BLASTn analysis. Twenty-seven (of 29) identified *H pylori*-sequences were amplified from pancreatic tissue samples. Sequences of *H.* sp. flexispira (n = 7) and *H. cinaedi* (n = 2) were only detectable in pancreatic tumor specimens (Table 3). Seven (of 23) PC patients were *helicobacter*-positive in gastric and/or duodenal samples, but only one of them was identified as *H pylori*. Eight PCR-products related to enteric *Helicobacter* spp. (*H. bilis*, n = 6; *H. hepaticus*, n = 2) were identified in gastric and duodenal specimens from 7 pancreatic cancer patients (*H. bilis* was detected in the stomach as well as duodenum in one PC patient) (Table 3). Five patients (four with PC and one with NE) were simultaneously *Helicobacter* DNA-positive in the pancreas and the stomach and/or duodenum. Four patients had different species in the different tissues, whereas one duodenal sample of a MEN-patient with *H pylori* DNA in the pancreas demonstrated both *H. pylori* and *H. hepaticus* in the DGGE analysis (Figure 1, patient no. 7). Agarose- and DGGE gel images as well as the result of DNA-sequencing of pancreatic as well as gastric and/or duodenal samples of 7 patients are shown in Figure 1.

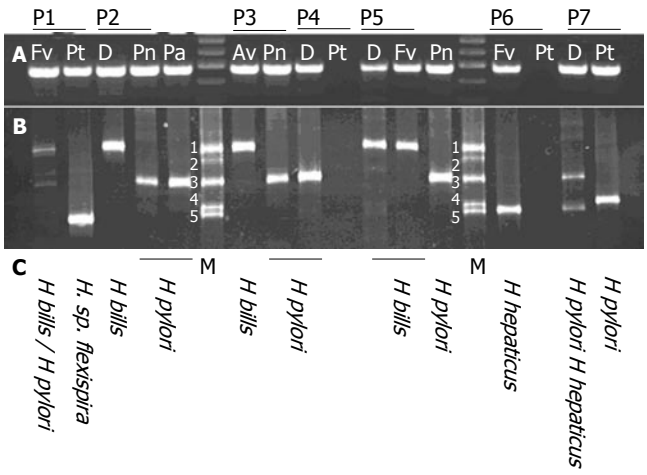


Figure 1 Agarose gel electrophoresis (**A**), DGGE (**B**), and the outcome of a public database comparison of *Helicobacter* 16S ribosomal DNA PCR-product sequences (**C**). From 7 pancreatic cancer patients (P), amplicons from the duodenum (D), fundus ventriculi (Fv), and antrum ventriculi (Av) were compared to amplicons from normal (Pn), atrophic (Pa) and pancreatic tumor (Pt) tissues. Migration markers (M) were (from top to bottom) amplified from DNA of H. bilis (1), *H. pullorum* (2), *H pylori* (3), *H. sp. flexispira* (4) and *H. hepaticus* (5).

DISCUSSION

We analyzed the prevalence of *Helicobacter* species ribosomal DNA by PCR in paraffin-embedded pancreatic and gastroduodenal samples from patients with pancreatic cancer and tissues of benign pancreatic diseases as well as controls of normal pancreas (from choledochus and colon cancer patients). *Helicobacter* species DNA was identified in the pancreas of 75% of the PC patients, 57% of the NE patients, 38% of the MEN patients, and 60% of the patients with chronic pancreatitis. Other benign pancreatic diseases and the normal pancreas controls were all *helicobacter*-negative (Table 1). Detection of *Helicobacter* species in pancreas is thus related to patients suffering from pancreatic cancer and chronic pancreatitis. Serological studies have previously demonstrated an association between serum antibodies to *H pylori* and pancreatic cancer[15, 16]. Moreover, *H pylori* increases the severity of tissue inflammation and production of proinflammatory cytokines in a rat model of ischemia/reperfusion-induced pancreatitis[22].

The distribution of *Helicobacter* ribosomal DNA in tumor and normal tissue of pancreatic cancer patients was also studied. *Helicobacter* species was commonly detected in tumors (48% of PC, 57% of NE, and 38% of MEN). The prevalence of *helicobacter* was much lower in the normal pancreas surrounding NE and MEN (5%) than surrounding PC (36%) (Table 2). There are at least two possible explanations. One is that *helicobacter* bacteria and/or *helicobacter* DNA may be taken up and retained by the diseased tissues such as tumors, the other is that *helicobacter* cells and/or cell debris in pancreas may be implicated in the genesis of PC and are therefore also found in the non-tumor pancreas, while in NE and MEN *helicobacters* may be an epiphenomenon and are thus uncommon in the non-tumor pancreas. Similar to the findings in normal tissues surrounding tumors in PC patients compared with NE and MEN, we have previously detected *Helicobacter* species in liver tissue

surrounding primary liver carcinoma but not colorectal liver metastases[23]. Hence, a possible participation of *Helicobacter* species in the genesis of exocrine pancreatic and liver carcinoma has to be further explored. Moreover, PCR detection of *H pylori* in the liver is associated with cirrhosis in hepatitis C patients with or without hepatocellular carcinoma[24], in analogy to *H pylori*-associated tissue inflammation in chronic atrophic gastritis progressing to gastric cancer[7, 25].

A majority of the PCR-products from pancreatic cancer samples are related to 16S ribosomal DNA sequences of *H pylori* previously identified in gallbladder tissue samples[26], and to *H.* sp. ´liver´, a putative subspecies of *H pylori* with liver tropism, detected in hepatocellular carcinoma[10], supporting the hypothesis of a *H pylori* subpopulation with hepatobiliary tropism[27]. We also identified *H. cinaedi* and *H.* sp. flexispira taxon 8 in some exocrine pancreatic cancer samples (Table 3). These species with a broad mammal host-range have been isolated from patients with bacteremia and gastroenteritis[28].

A low prevalence of *H pylori* DNA was found in gastric and duodenal samples from the patients in this study (Table 3). At least half of the adult human population is infected with *H pylori*[7], but only 14% of the PCR-products from the stomach of patients with exocrine pancreatic cancer could be identified as *H pylori*. However, *H. bilis* has been identified in 60% of *helicobacter*-positive gastroduodenal specimens. *H. bilis* is associated with chronic hepatitis and chronic enteric inflammation in susceptible laboratory mouse strains[28] and has been recently identified in both diseased human gallbladder and bile by PCR-based methods[12]. To our knowledge, *H. bilis* has not previously been detected in tissue samples of the human stomach and small intestine. The reason why the detection rate of *H pylori* is low in the stomach and duodenum of PC patients remains obscure. This may partly be explained by the fact that most or all patients were given metronidazole preoperatively.

We analyzed stomach and duodenum tissue specimens from pancreatic cancer patients to detect whether gastric *Helicobacter* species, such as *H pylori*, may disseminate to the pancreas in pancreatic cancer patients. However, DNA of different *Helicobacter* species in the pancreas compared with gastroduodenal tissue was identified in patients who were *Helicobacter*-positive both in the stomach and pancreas (Figure 1). Moreover, many pancreas-positive PC patients were negative in stomach samples and vice versa, not supporting migration of *helicobacter* microorganisms colonizing the stomach to the pancreas in the studied PC patients.

B. fragilis constitutes a part of the indigenous microflora of the human gut, predominantly of the colon. A nested PCR-assay for the genus *Bacteroides* was designed to study whether a major constituent of the normal microflora of the lower bowel could translocate to a diseased pancreas. None of the pancreatic tissue specimens was positive for *Bacteroides* spp., suggesting that bacterial translocation from the bowel to pancreas does not frequently occur in patients with cancer or a benign pancreas disease. However, the indigenous microflora may more easily be washed off tissues during sample preparations such as paraffin deembedding. Putative pathogens, such as some *Helicobacter* species, might be more closely associated with the gut mucosa.

Chronic inflammation is a characteristic feature of gastric-, colon- and hepatobiliary tract cancers[29]. Bacterial cell-wall peptidoglycan and lipopolysaccharide can stimulate the human innate immunity and induce inflammation. Bacterial DNA, so called CpG motifs, has been shown to activate macrophages, neutrophils as well as cell migration and to induce B cell activation and hyper-IgM production in patients with primary biliary cirrhosis[30, 31]. Proinflammatory cytokines, reactive oxygen species and other inflammatory mediators are associated with a chronic *Helicobacter*-induced tissue inflammation[25]. Such factors probably increase genomic DNA damage and cell proliferation as well as inactivate tumor-suppressor genes, events also associated with malignant transformation of pancreatic cells[1, 6, 32]. Tumor-associated chronic inflammation[29] may be induced and maintained by bacteria or bacterial cell debris, originating from *helicobacter* and/or other microbial species.

In conclusion, 16S ribosomal DNA of gastric *H pylori* and some enteric *Helicobacter* species is commonly detectable in tissue samples from patients with pancreatic cancer but not from controls. To further explore a possible role of gastric and enterohepatic *Helicobacter* species in pancreatic malignancy, more studies of pancreatic cancer and the emerging *Helicobacter* genus and related organisms are needed.

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BASIC RESEARCH

Correlation between TIMP-1 expression and liver fibrosis in two rat liver fibrosis models

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Abstract

AIM: To evaluate serum TIMP-1 level and the correlation between TIMP-1 expression and liver fibrosis in immune-induced and CCL_4-induced liver fibrosis models in rats.

METHODS: Immune-induced and CCL_4-induced liver fibrosis models were established by dexamethasone (0.01 mg) and CCL_4 respectively. Serum TIMP-1 level was detected with ELISA, while histopathological grade of liver biopsy was evaluated. Spearman rank-correlation test was used to analyse the difference of the correlation between the TIMP-1 expression and hepatic fibrosis in the two fibrosis models. Furthermore, *in situ* hybridization was used to determine the expression difference of TIMP-1 mRNA in the two models.

RESULTS: Positive correlation existed between serum TIMP-1 level of immune induced group and the histopathological stages of fibrosis liver of corresponding rats (Spearman rank-correlation test, $r_s = 0.812$, $P < 0.05$), and the positive *in situ* hybridization signal of TIMP-1 mRNA was strong. In CCL_4-induced liver fibrosis model, the correlation between the serum TIMP-1 level and the severity of hepatic fibrosis was not statistically significant(Spearman rank-correlation test, $r_s = 0.229$, $P > 0.05$). And compared with immune-induced model, the positive *in situ* hybridization signal of TIMP-1 mRNA was weaker, while the expression variation was higher in hepatic fibrosis of the same severity.

CONCLUSION: The correlations between TIMP-1 expression and liver fibrosis in two rat liver fibrosis models are different. In immune-induced model, serum TIMP-1 level could reflect the severity of liver fibrosis, while in CCL_4-induced model, the correlation between the serum TIMP-1 level and the severity of hepatic fibrosis was not statistically significant.

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Key words: TIMP-1; Liver fibrosis; Models, rat; Immune-induced, CCL_4-induced; Serum; Tissue of liver

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INTRODUCTION

Hepatic fibrosis is a pathological process with the net deposition of extracellular matrix (ECM) proteins. The change of ECM is mainly regulated by matrix metalloproteinases (MMPs), which are a family of proteolytic enzymes that are capable of degrading the ECM[1-3]. The activity of MMPs is tightly regulated by the amount of active protein and the concentration of specific inhibitors, called tissue inhibitors of metalloproteinases (TIMPs)[4,5]. Extensive studies have identified that TIMPs play a key role in the progression of fibrosis. TIMP-1 is the first discovered tissue inhibitor of TIMPs in liver which can inhibit most interstitial collagenases and MMP-9. Murawaki *et al* revealed that serum level of TIMP-1 could reflect the change of liver TIMP-1 in patients with chronic liver disease and liver TIMP-1 concentration increases with progression of the liver disease[6-9]. So, TIMP-1 has been considered as a useful diagnostic index of hepatic fibrosis[10-12]. Our previous studies[13-15] also concentrated on the establishment of the new hepatic fibrosis diagnostic index. Interestingly, in animal experiments we found the expression of TIMP-1 in liver tissue has remarkable difference in different rat liver fibrosis models[13,14]. In the present experiment, the serum TIMP-1 level of experimental rats of liver fibrosis model was detected with ELISA and association with histopathological grading of fibrosis liver was analyzed. Furthermore, *in situ* hybridization was used to analyze the expression difference of TIMP-1 mRNA between the two models.

MATERIALS AND METHODS

Animals

One hundred and twenty adult female Wistar rats (provided by Experimental Animal Center of Fourth Military Medical University, Xi'an, China) weighing 150-180 g, fed with common stuff and water, were employed in the study (Approval for this project was obtained from the Institutional Review Board at Fourth Military Medical University). Immune-induced rat liver fibrosis model was established according to the method of Wang *et al*[16]. Dexamethasone (0.01 mg) was injected into the rats through coccygeal vein in one hour after the first and second injection of human serum albumin (HAS). For CCL_4 group, 55 rats were injected with 400 mL/L CCL_4 in peanut oil subcutaneously at a dose of 3 mL/kg twice (Monday and Thursday) weekly, totally 10 wk[17]. Ten normal rats were served as control group.

Specimen preparation

From the first Sunday in the process of model induction (for immune induced group, from the first coccygeal vein injection of HSA), 5 rats from each group were selected randomly on Sunday, until all rats were involved. Rats taken out from immune induced group on the first Sunday were numbered IW1A to IW1E (Immune-induced rat liver fibrosis model wk 1st A-E), while the rats from CCL_4 induced group were numbered CW1A to CW1E (CCL_4-induced rat liver fibrosis model wk 1st A-E). All rats were immediately sacrificed under narcosis after taken out. Following decapitation 2 mL blood was collected from each rat. Liver was quickly excised, and rinsed in ice-cold saline. Ten rats in control group were treated in the same way after 10 wks' feeding. Blood specimen was centrifuged, and the serum was obtained, and stored at -70℃. Livers were cut into 1 mm × 1 mm × 1 mm blocks. Part of it was fixed in 40 g/L formaldehyde, and part was fixed in 25 g/L glutaraldehyde. All liver specimens were stored at -70℃. Specimens were treated all together after collected.

Pathologic observation and histopathological grading of fibrotic liver tissue

Liver sections were processed together for routine hematoxylin-eosin (HE) stain. Pathological diagnosis of each liver specimen was assessed and graded from 0 to VI by three pathologists in a blinded manner (fibrotic stage was determined when more than two pathologists reached the same diagnosis; if inconsistent results were reached among the three pathologists, the fourth determined the final diagnosis) according to the criteria described by Wang *et al*[18] (Table 1).

ELISA detection of serum TIMP-1

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the serum TIMP-2 level[19,20]. Mouse TIMP-2 monoclonal antibody was purchased from American Maxim Company (MAB-0283) and test was performed according to the protocol described previously[21]. The absorbance of serum TIMP-2 was determined at 450 nm with an ELISA reader (EL × 800, Bio-Tek Instruments. Inc, USA).

Table 1 Scoring system assessing the degree of fibrosis

Degree of fibrosis	Score
No fibrosis	0
Slight fibrosis expanding to some portal areas and central veins	I
Marked fibrosis expansion, but without portal to portal bridging	II
Fibrosis expanding to most portal areas with occasional portal to portal bridging	III
Pseudolobules formed and partly replacing the normal architecture of the liver lobules	IV
Occasional pseudolobules formed (incomplete cirrhosis)	V
Congested with pseudolobules, and between pseudolobules wide hyperplastic collagen fiber existed (complete cirrhosis)	VI

In situ hybridization of TIMP-1 mRNA in tissue of fibrosis liver

Paraffin sections were hybridized with digoxingenin-labelled cRNA probes as described by Wang *et al*[22]. The cRNA probe for TIMP-1 and the *in situ* hybridization kit were purchased from Boshide Biological Technology Limited Company, Wuhan, China (No MK1549). Expression of TIMP-2 mRNA was assessed quantitatively by image pattern analysis with gray scale scanning. Control was performed with normal liver tissue.

Specimen preparation for transmission electron microscopy

Specimens were routinely prepared for transmission electron microscopy (TEM) observation and examined under a JEOL JEM-100CXII electron microscope.

Statistical analysis

Spearman rank-correlation test, LSD-*t* and Student's *t* test were used to analyze the results. $P < 0.05$ was taken as significant.

RESULTS

Comparison of histopathology between immune induced and CCL_4 induced liver fibrosis groups

Distinct difference of histopathological changes presented between the liver fibrosis of immune group and CCL_4 group. The liver fibrosis in immune group rat was characterized by portal fibrosis with marked ductular proliferation extending to fibrous septa (Figure 1A, specimen from IW10E). Pseudolobules formed and partly replaced the normal architecture of the liver lobules, but the fibrotic septum was small and sparse. In CCL_4 group, prominent fatty degeneration and necrosis were found and the normal architecture of the liver lobules was markedly destroyed, most of which were replaced by pseudolobules (Figure 1B, specimen from CW10A). Compared with immune induced group, fibrotic septum in CCL_4 group was wide and compact.

Histopathological grading

All rats in immune induced group survived the experiment. In tissue specimens of IW1A-E, only slight hepatocyte swelling could be observed, whereas no obvious damage

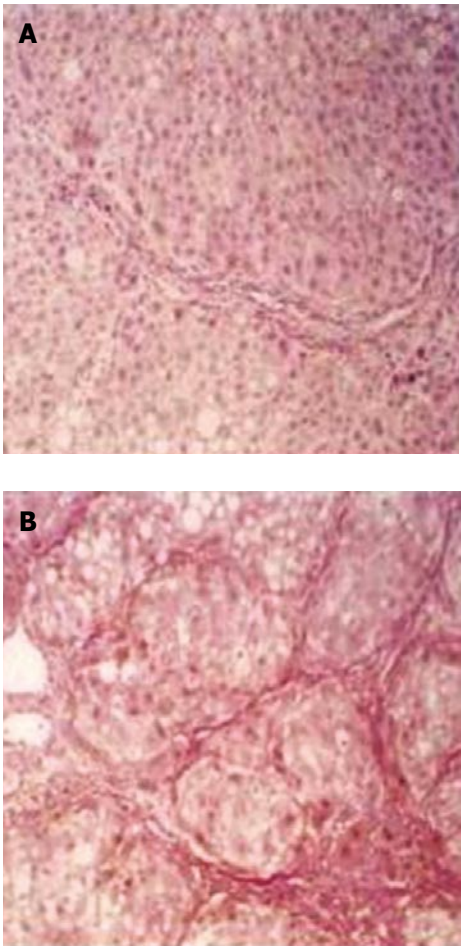


Figure 1 Light microscope observation of rat liver fibrosis (HE ×100). **A**: immune-induced liver fibrosis; **B**: CCl_4-induced liver fibrosis.

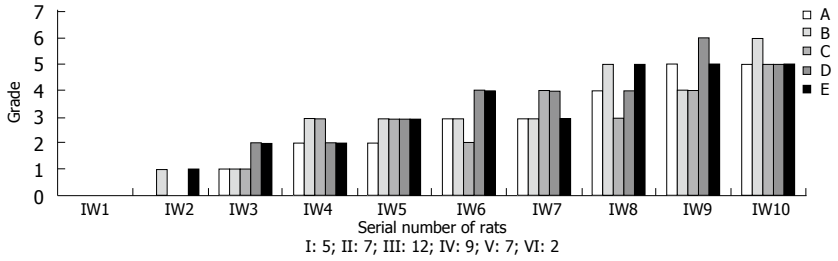


Figure 2 Histopathological grade of fibrosis of immune induced group.

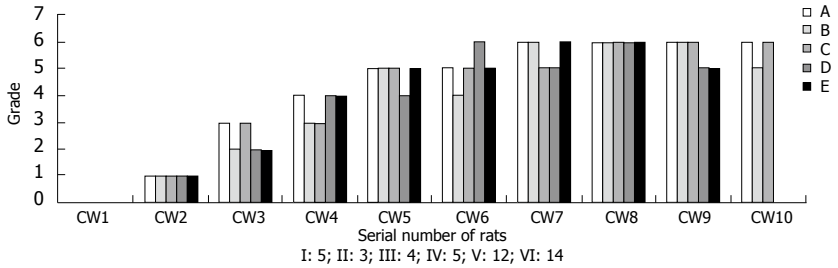


Figure 3 Histopathological grade of fibrosis of CCL_4 induced group.

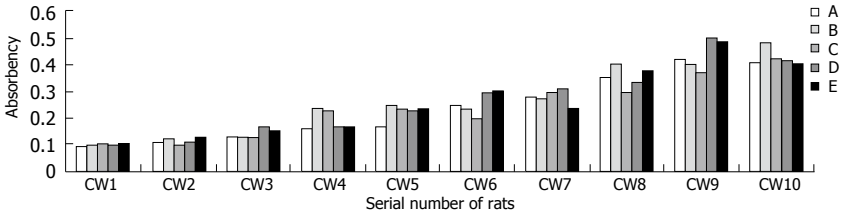


Figure 4 ELISA results of serum TIMP-1 of immune induced group.

presented. Histopathological stages of the five specimens were all determined to be stage 0. Among the specimens of IW2-5, obvious hyperplasia of connective tissues could be detected, but most of the histopathological stages were not above III. Among rats of immune induced group taken out after fifth week, with induction time extending, more livers were determined to be stage IV and V fibrosis. However, specimens determined to be stage VI were few (Only two fibrosis liver specimens of rats taken out at tenth week were determined to be stage VI) (Figure 2). Seven rats in CCL_4 induced group died in the process of experiment (5 rats died before wk 5 and 2 died at wk 7), which livers were immediately excised and frozen at -70℃. Histopathological observation of the died rats showed severe liver damages existed in the liver such as inflammation, necrosis, excessive collagen deposition and inflammatory cells infiltration, *etc*. Obvious damage already existed in the liver tissue of the five rats of CW1, but no fiber hyperplasia presented. Histopathological stages were stage 0 according to the criteria described above. Specimens of CW2 presented obvious hepatocyte degeneration and necrosis, and vacuole degeneration and inflammatory cell infiltration also could be observed, but fiber hyperplasia was not predominant. The histopathological stages were determined to be stage I. From wk 3, normal architecture of the liver was rather lost, replaced with volumes of connective tissue. Hereafter, with the passage of time, fibrotic separations increased between the portal areas or between the portal area and the central vein, leading to the increasing formation of pseudolobules, and histopathologically stage IV or V was determined. In most of the rats taken out after wk 7, liver tissues were congested with pseudolobules and between the pseudolobules large numbers of collagens deposited, which were classified as classic stage VI (Figure 3).

ELISA reading of serum TIMP-1

In theory, reading (absorbency) of ELISA reader correlates with serum TIMP-1 level, which could properly reflect the quantity of serum TIMP-1[23,24].

Results of serum TIMP-1 level of immune induced group and CCL_4 induced group are presented in Figures 4 and 5 respectively. Data of control group accorded with normal distribution (mean = 0.105, 95% percentile = 0.137).

Positive correlation existed between serum TIMP-1 level of immune induced group and the histopathological stages of fibrosis liver of corresponding rats (Spearman rank-correlation test, $r_s = 0.812$, $P < 0.05$), indicating serum TIMP-1 level could be considered as an index reflecting the degree of liver fibrosis.

No clear correlation between the serum TIMP-1 level

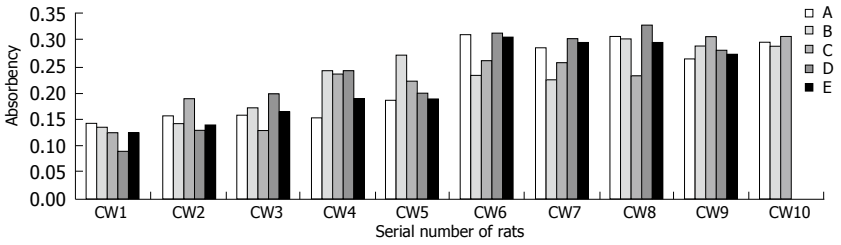


Figure 5 ELISA results of serum TIMP-1 of CCl_4 group.

Table 2 Results of image pattern analysis of expression of TIMP-1 mRNA

Group	*n*	TIMP-1 mRNA
Immune group[a]	7	421.90 ± 19.00
CCL_4 group[c]	7	188.80 ± 62.80
Control group	10	52.75 ± 4.25

[a]$P < 0.05$ compared with CCL_4 group, $t = 9.398$; [c]$P < 0.05$ compared with control group, $t = 3.414$.

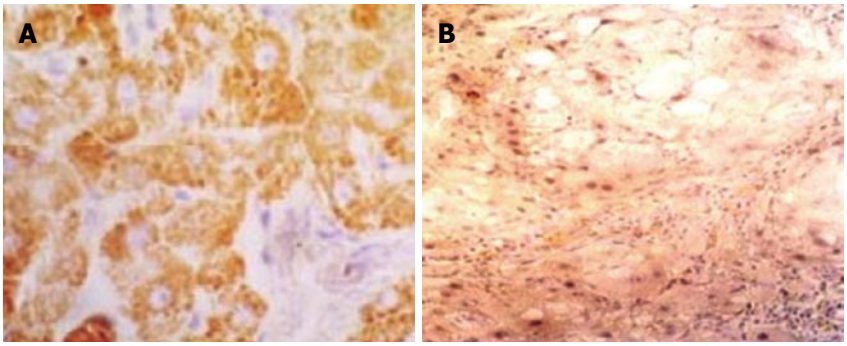


Figure 6 Expression of TIMP-1 mRNA in tissue of rat fibrosis liver. **A**: Immune-induced liver fibrosis (× 400); **B**: CCL_4-induced liver fibrosis (× 200).

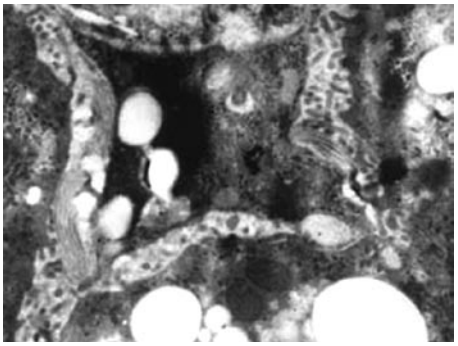


Figure 7 Activated HSC in immune induced rat liver fibrosis (× 10000).

of CCL_4 induced group and the histopathological stages of corresponding rats (Spearman rank-correlation test, $r_s = 0.229$, $P > 0.05$), indicating serum TIMP-1 level of CCL_4 group could not reflect the degree of liver fibrosis. Even so, the serum TIMP-1 level of CCL_4 group was obviously higher than that of control group (LSD-*t*, $P < 0.001$).

Expression of TIMP-1 mRNA in tissue of fibrosis liver

Seven specimens from immune group and CCL_4 group respectively, which were all determined as stage V histopathologically (for CCL_4 group, the seven specimens were randomly selected from the 12 stage V specimens), were used to compare the difference of TIMP-1 mRNA expression. Ten specimens of control group were detected at the same time. In the fibrosis liver of immune group, expression of TIMP-1 mRNA was detected in myofibroblasts, fibroblasts and vascular endothelial cells, especially predominant in the portal areas and fibrotic septum. The positive signals of TIMP-1 mRNA located in cytoplasm as brown particles, but not found in nucleus (Figure 6A). For CCL_4 group, myofibroblasts, fibroblasts in the portal area and vascular endothelial cells expressed TIMP-1 mRNA. However, compared with immune group, the signals were weaker (Figure 6B).

Image pattern analysis of *in situ* hybridization revealed that among immune, CCL_4 and control groups, expression of TIMP-1 mRNA of immune group was highest ($t = 9.398$, $P < 0.05$); while between CCL_4 group and control group, expression of TIMP-1 mRNA of CCL_4 group was obviously higher ($t = 3.414$, $P < 0.05$) (Table 2). The standard deviation of data of CCL_4 group was distinctly higher than immune group (62.80 > 19.00), indicating greater variation existed in the specimens of CCL_4 group even with the same histopathological stage.

Transmission electron microscope examinations

In liver fibrosis of immune group, large quantities of activated hepatic stellate cells (HSC) were detected. In the cytoplasm of activated HSCs, there were abundant rough endoplasmic reticulum, but fatty droplets were obviously decreased (Figure 7). Around the activated HSCs, in Disse space, between hepatocytes, and surrounding areas of bile duct, dense deposition of collagens could be found. Portal areas extended, where great number of myofibroblasts existed. In denatured hepatocytes, many swelling mitochondria and areas of fatty drops existed. For CCL_4 group, severe collagen deposition could be observed, but with fewer activated HSCs.

DISCUSSION

Hepatic fibrosis is a consequence of different chronic liver diseases caused by hepatitis viruses, drugs, alcohol, parasite, and autoimmune mechanism. Nowadays there are several kinds of animal model of liver fibrosis for the study of the mechanisms of liver fibrosis, among which CCL_4-induced rat liver fibrosis model and immune-induced rat liver fibrosis model were extensively investigated and applied[25]. Fibrosis caused by different pathogens has different pathogenesis processes. Therefore, not every fibrosis model is universally suitable. Which model should be applied in a given study is of great importance. But now, there is still no clear guideline for this.

Over the past 15 years, substantial progress has been made in understanding the role of TIMPs (TIMP-1 and TIMP-2) in the regulation of hepatic fibrosis. Hepatic

fibrosis is a pathological process with the net deposition of ECM proteins[26]. The change of ECM is mainly regulated by MMPs. MMPs are a family of proteolytic enzymes that are capable of degrading the ECM and the activity of MMPs is tightly regulated by the amount of active protein and the concentration of TIMPs[27]. It has been identified that TIMP-1 plays a predominant role in regulating fibrosis by inhibiting the activity of MMP-1, MMP-3, MMP-9, and TIMP-1 is far stronger than TIMP-2[28]. So, TIMP-1 has been considered as an important factor in the progression of liver fibrosis and the expression level could be used as a new index of fibrosis severity.

In our previous studies[13,14], we found that the expression of antigen and mRNA of TIMP-1 in liver tissue of CCL_4-induced fibrosis rat was weaker compared with immune-induced fibrosis. Although higher than normal rat liver, the expression of TIMP-1 of CCL_4-induced liver fibrosis was not consistent with the severity of hepatic fibrosis. In the current study, the fibrosis in CCL_4-induced rat liver fibrosis model developed fast and almost all the histopathological grading of the rats which survived the experiment in this group belonged to grade V, and VI. Fibrous tissue in the fibrosis liver of this model was presented as wide and compact fibrotic septum, and most of the normal architecture of the liver lobules were destroyed and replaced by pseudolobules. But, *in situ* hybridization and immunohistochemical staining showed mRNAs and antigens of TIMP-1 expressed weakly, not proportional to the severity of the fibrosis. While, in immune-induced rat liver fibrosis model, even the fibrotic septum were small and sparse and histopathological grading was lower, the expression of mRNAs and antigens of TIMP-1 was higher compared with CCL_4-induced rat liver fibrosis model. We used Spearman rank-correlation test to analyse the correlation between the serum levels of TIMP-1 and the histopathological stages of corresponding rat, and found that no clear correlation existed between the serum levels of TIMP-1 and the histopathological stages of corresponding CCL_4-induced rat. While, serum levels of TIMP-1 of immune-induced fibrosis liver could reflect the severity of liver fibrosis. In the process of experiment, we failed to detect clear pathologic progression in the inducing process of CCL_4, because the pathological changes developed very fast. We also analyzed the results of *in situ* hybridization of TIMP-1 mRNA in CCL_4-induced fibrosis liver, which indicates that even with same histopathological stage, the TIMP-1 mRNA expression varied greatly, which was distinct from immune-induced rat liver fibrosis.

Currently studies on fibrosis are mostly focused on chronic hepatitis caused by hepatitis virus infection, which is a chronic progressive process. Our study revealed that the fibrosis induced by CCL_4 progressed fast, and autopsies of the fibrosis livers presented hepatocyte vacuole degeneration and necrosis at the early period of CCL_4 induction, indicating the subsequent ECM deposition might be the recovery process, during which connective tissue replaced the damaged architecture. The nosogenesis of CCL_4 induced fibrosis may contribute to this change[29]. The hepatotoxicity of CCL_4 results from its metabolites, the free radicals CCL_3^-, which could damage the hepatocyte by causing lipid peroxidation and by binding covalently to cell structures, leading to flaky hepatocyte vacuolar degeneration and necrosis. This is of obvious difference from the known fibrosis mechanism of viral hepatitis (HBV and HCV), which has been considered as a consequence of immunologic injury caused by the virus infection.

The fibrosis of immune-induced rat model is caused by type III allergy (immune complex type)[30]. The severity of the fibrosis is not so much high as CCL_4-induced rat liver fibrosis, while the progression is similar to human viral hepatitis. Immune complex in serum exerts its hepatotoxicity through activating HSCs that can express TIMP-1, which enhances the deposition of ECM through inhibiting the activity of MMPs[31]. Under TEM, larger numbers of activated HSCs supported the above hypothesis.

In conclusion, the pathological changes of immune-induced rat liver fibrosis model is a gradual process and serum TIMP-1 level can reflect the severity of fibrosis. CCL_4-induced rat liver fibrosis model develops fast in comparison with immune-induced rat liver fibrosis model and no significant correlation exists between the serum TIMP-1 level and the severity of fibrosis, both indicating that the model is unsuitable for experimental observation. Taken together of the difference of the two models, we think immune-induced rat liver fibrosis model is more suitable for the study of human chronic viral hepatitis.

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BASIC RESEARCH

An efficient method of sorting liver stem cells by using immuno-magnetic microbeads

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Abstract

AIM: To develop a method to isolate liver stem cells fast and efficiently.

METHODS: Fetal mouse liver cells were characterized by cell surface antigens (c-Kit and CD45/TER119) using flow cytometry. The candidate liver stem cells were sorted by using immuno-magnetic microbeads and identified by clone-forming culture, RT-PCR and immunofluorescence assays.

RESULTS: The c-Kit$^-$(CD45/TER119)$^-$ cell population with 97.9% of purity were purified by immuno-magnetic microbeads at one time. The yield of this separation was about 6% of the total sorting cells and the cell viability was above 98%. When cultured *in vitro* these cells had high clone-forming and self-renewing ability and expressed markers of hepatocytes and bile duct cells. Functionally mature hepatocytes were observed after 21 d of culture.

CONCLUSION: This method offers an excellent tool for the enrichment of liver stem cells with high purity and viability, which could be used for further studies. It is fast, efficient, simple and not expensive.

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Key words: Liver stem cells; Immuno-magnetic microbeads; Sorting; Flow cytometry

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INTRODUCTION

Stem cell is a very small population of cells in various tissues. It is a self-renewing, typically multipotent progenitor with the broadest developmental potential in a particular tissue at a particular time[1,2]. Studies of stem cell have yielded much exciting insight into the field of life science and medicine including cancer, transplantation and proteomics.

Large-scale proteomic study of stem cell, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry analysis, requires relatively large amounts of protein sample extracted from the cells[3]. For the low-abundance samples such as stem cells, the key to such study relies on the successful obtaining of enough amounts of stem cells with high purity. Two most commonly used methods, magnetic activated cell sorting (MACS) and high-speed fluorescence-activated cell sorting (FACS), have been employed to isolate and purify a special population of cells based on the cell surface markers. In the latter method, however, there are some disadvantages. For example, a high fluid pressure might damage the biological activity of the sorting cells; for rare cells such as stem cells, to isolate large amounts of them is also time-consuming[4].

The liver is the central organ of nutrient digestion and processing, where most of a person's metabolism occurs. Although liver regeneration is not dependent on a small group of progenitor or stem cells, which may appear in large numbers when mature hepatocytes are inhibited from proliferation[5], it is believed that in a growing liver both hepatocytes and cholangiocytes differentiate from a common progenitor, or liver stem cell[6]. To date, few liver stem cell-specific markers are available, and some are also shared by hematopoietic progenitors[7,8]. Recently, by using FACS liver stem cells have been isolated based on some non-specific cell surface markers (c-Kit, CD45, TER119, c-Met and Dlk) and identified[6,9-11]. In the study reported here, using immuno-magnetic microbeads, we sorted large amounts of candidate liver stem cells with high purity and identified them with self-renewing capability and multilineage differentiation potential.

MATERIALS AND METHODS

Mice and cells

Animal experiments involving mice were approved by the Institute's Animal Care and Use Committee. BALB/c mice were maintained under pathogen-free conditions. Day 0 of gestation was taken to be detection of a vaginal plug after overnight mating. Embryos were dissected and fetal liver cell suspensions were prepared by mechanical dissociation and then passed through a 30 μm nylon mesh to remove clumps.

FACS analysis and cell sorting

Dissociated liver cells were incubated at 4 ℃ for 10 min with biotinylated anti-CD45.2, TER119 monoclonal antibodies (mAbs) (eBioscience, San Diego, CA). After three washes with staining medium (PBS containing 3% FBS), cells were incubated with FITC-conjugated anti-c-Kit mAb (eBioscience), and streptavidin-labeled phycoerythrin (eBioscience) at 4 ℃ for 10 min. Finally, cells were washed three times and resuspended in staining medium. The labeled cells were analyzed and sorted with FACSAria (Becton Dickinson). The acquisition rate was set at 35 000 events per second. Gating was implemented based on negative control staining profiles.

Immuno-magnetic sorting

Dissociated fetal liver cells were first stained with biotinylated anti-CD45.2, TER119 and/or c-Kit mAbs at 4 ℃ for 10 min. Cells were washed twice with PBS containing 0.5% BSA and 2 mmol/L EDTA. Enrichment of target cells by magnetic microbeads was carried out according to the manufacturer's (Milteny Biotec, Bergisch Gladbach, Germany) protocol. Briefly, cells were resuspended in 80 μL of the same buffer per 10^7 total cells and incubated with anti-biotin microbeads for 15 min at 6-12 ℃. Cells were washed twice and finally resuspended in 500 μL of buffer per 10^7 total cells. A pre-moistened MS column was placed in the magnetic field of a suitable MACS separator. The cell suspension was applied onto the column and then washed three times. Effluents were collected as the negative fraction. Magnetically labeled cells as the positive fraction were collected by flushing the column with buffer after the column was placed outside of the magnetic field. The sorted cells were then incubated at 4 ℃ for 10 min with phycoerythrin-streptavidin and analyzed with FACSCalibur (Becton Dickinson).

Cell culture

Cell culture was performed as described previously with minor modifications[9,10]. Liver cell populations were plated onto 20 μg/mL laminin-coated (Gibco BRL, Gaithersburg, MD) 24-well plates or chamber slides at a density of 1000 cells/cm^2 and maintained at 37 ℃, with 50 mL/L CO_2 for 8-21 d. The medium was DMEM/F12 supplemented with 10% FCS, 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 U/mL penicillin-streptomycin, 1 × nonessential amino acids (all from Gibco BRL), 1 μg/mL human insulin and 10^{-7} mol/L dexamethasone (Sigma-Aldrich, St. Louis, MO) renewed every 4 d. Mouse recombinant hepatocyte growth factor (40 ng/mL) and OSM (10 ng/mL) (R&D system, Minneapolis, MN) were added 24 h after culture initiation.

RT-PCR

Detection of hepatocyte or cholangiocyte marker gene expression by RT-PCR was conducted as described[12]. After treated with DNase I (Promega, Madison, WI), equal amounts of RNAs were reverse-transcribed and the cDNAs were amplified by the PCR with gene-specific primers at 94 ℃ for denaturing, 54 ℃ for annealing, and 72 ℃ for extension. β-actin mRNA was used as an internal control. Previous experiments determined 28 cycles to be optimal. The gene-specific intron-spanning primers used are as follows: α-fetoprotein (AFP, amplification of 821 bp), sense 5'-TCACACCCGCTTCCCTCATCCT-3', antisense 5'-CATCCTGCAGACACTCCAG-3'; albumin (ALB, amplification of 705 bp), sense 5'-AGAAGACACCCTGATTACTCT-3', antisense 5'-TCGAGAAGCAGGTGTCCTTGT-3'; cytokeratin 19 (CK19, amplification of 566 bp), sense 5'-GTCCTACAGATTGACAATGCTCG-3', antisense 5'-CTGATCACGCTCTGGATCTGTG-3'; β-actin, (amplification of 390 bp), sense 5'-GTCATCACTATTGGCAACGAGCG-3', antisense 5'-CTAGAAGCACTTGCGGTGCACG-3'. The amplified products were analyzed by electrophoresis on 1% agarose gels and stained with ethidium bromide.

Immunofluorescence assays

Cultured cells were washed with PBS and fixed and permeabilized with methanol/acetone (95:5) for 20 min at –20 ℃. They were then incubated with the primary Ab goat anti-mouse ALB (Bethyl, Montgomery, TX) and mouse anti-CK19 (DakoCytomation, Denmark) in a moist chamber for 16 h at 4 ℃. After washing in PBS, cells were incubated with Cy3-conjugated rabbit anti-goat IgG (Sigma) and FITC-conjugated bovine anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h at 4 ℃. Nuclei were counterstained with 300 nmol/L DAPI in PBS for 5 min. After final washing, cells were observed using a laser scanning microscope.

Periodic acid-Schiff staining

Cultured cells were fixed in 4% phosphate-buffered paraformaldehyde and then oxidized in 1% periodic acid for 5 min, rinsed in dH_2O three times, treated with Schiff's reagent for 15 min, rinsed again in dH_2O, stained with Mayer's hematoxylin for 1 min, and finally rinsed in dH_2O.

RESULTS

Flow cytometric fractionation and sorting of fetal mouse liver cells

Embryo fetal liver contains lymphohematopoietic progenitors characterized by the CD45 and TER119 antigens. Double staining of embryonic day (ED) 12 fetal mouse liver cells with anti-(TER119 + CD45) mAb and anti-c-Kit (also CD117) mAb revealed four cell populations (Figure 1A). The Q3 c-Kit^-(CD45/TER119)$^-$ cell population, which had no obvious boundary but

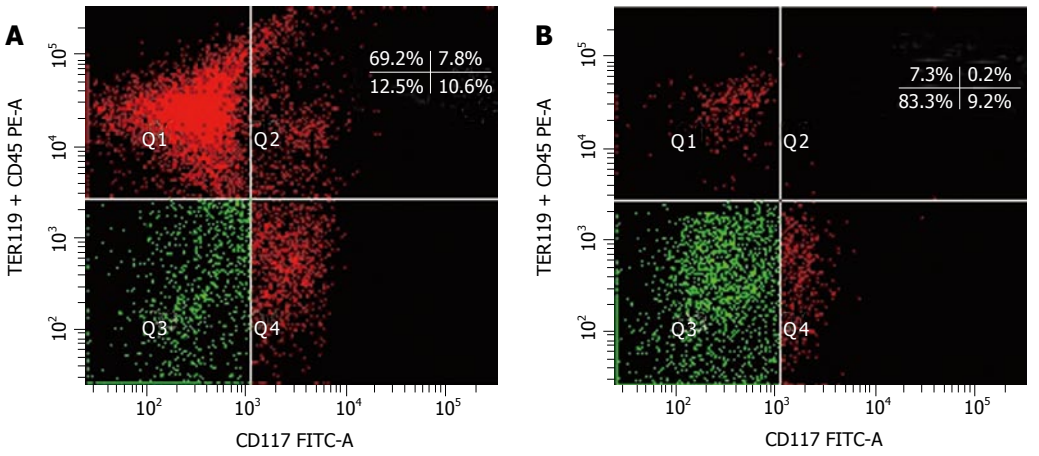


Figure 1 Flow cytometric fractionation and sorting of fetal mouse liver cells. **A**: Two-color FACS analysis of ED 12 mouse fetal liver cells by FACSAria. A mixture of biotinylated anti-CD45.2 and anti-TER119 mAb versus FITC anti-CD117 mAb defined the four populations of the dot plot. **B**: Re-analysis of the sorted Q3 cells.

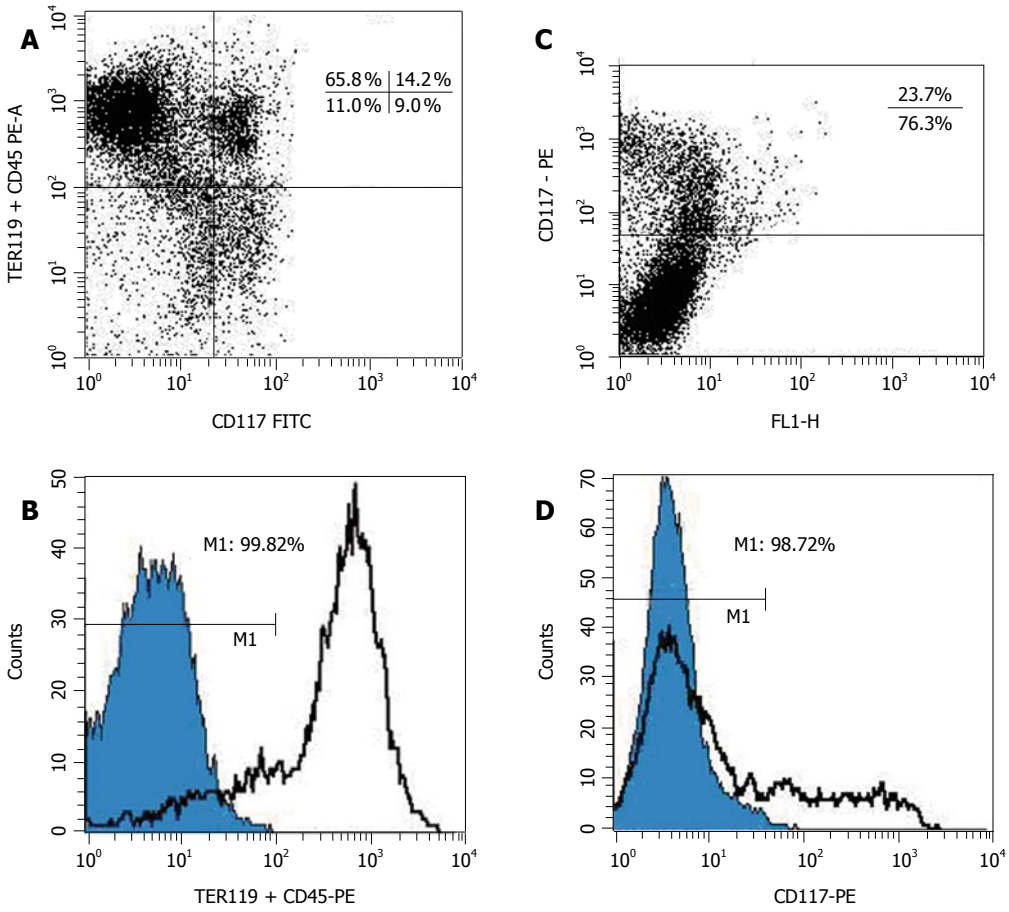


Figure 2 Flow cytometric analysis of fetal mouse liver cells before and after immuno-magnetic sorting. **A**: Two-color FACS analysis of ED 13 mouse fetal liver cells by FACSCalibur. Cells were stained as described in Figure 1A. **B**: Histograms for the immuno-magnetically sorted $(CD45/TER119)^-$ cells labeled with phycoerythrin-streptavidin (blue) and the total cell populations before sorting (white). **C**: FACS analysis of ED 13 mouse fetal liver cells stained with the biotinylated anti-c-Kit mAb. **D**: Analysis of the immuno-magnetically sorted $c\text{-}Kit^-$ cells labeled with phycoerythrin-streptavidin (blue). The histogram of Figure 2B is also shown (white).

continuous fluorescence distribution with the Q4 cell population, was considered to be liver stem cell or contain liver stem cell[9,10]. Flow cytometric sorting for Q3 cells revealed the purity of the sorted cells was 83.3% (Figure 1B). We observed that most of the non-$c\text{-}Kit^-$ $(CD45/TER119)^-$ cells were from Q4 cells near Q3 cell population.

Immuno-magnetic sorting of liver stem cells

ED 13 fetal mouse liver cells were analyzed in FACSCalibur (Figure 2A) and similar result was obtained to that shown in Figure 1A. The fraction of $(CD45/TER119)^-$ and $c\text{-}Kit^-$ cell population was 20% and 76.8%, respectively. Immuno-magnetic microbeads were used to separate these two cell populations. After double labeled with the biotinylated primary anti-mAb TER119 and CD45.2, which was then recognized by a monoclonal anti-biotin antibody coupled to microbeads, total fetal liver cells were applied onto the separation column in a magnetic field and only the unlabeled cells (negative fraction, both $TER119^-$ and $CD45^-$) were passed through. FACS analysis of the negative fraction revealed that more than 99% of the cells were $(TER119/CD45.2)^-$ (Figure 2B), indicating that the purity of the sorted cells was above 99%. To separate $c\text{-}Kit^-$ cell population from the liver cells, a biotinylated anti-c-Kit mAb was used. Total fetal liver cells stained with this mAb were first analyzed by FACS (Figure 2C) and the result was similar to that from FITC-labeled

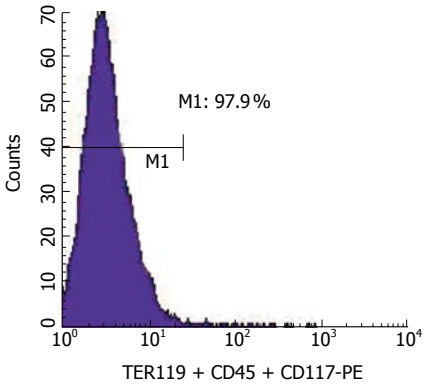


Figure 3 Analysis of the immuno-magnetically sorted c-Kit$^-$(CD45/TER119)$^-$ cells. After sorted, the negative fraction was stained with phycoerythrin-streptavidin and analyzed by FACS.

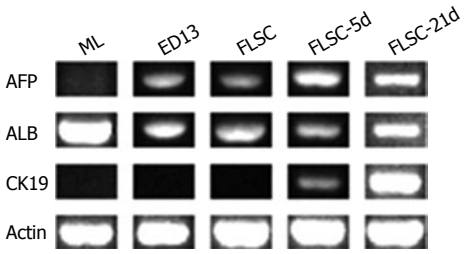


Figure 5 Hepato- and cholangiocyte- specific gene expressions of liver cells. The mRNA was prepared and RT-PCRs were performed for the genes displayed in the figure in each cell subset. β-actin (Actin) gene expression was used as controls for mRNA content per sample. A representative experiment for all the gene expressions of a total of three independent ones is shown. ML, mature mouse liver cells; ED13, ED 13 mouse fetal liver cells; FLSC, fetal liver stem cells [c-Kit$^-$(CD45/TER119)$^-$ cells]; FLSC-5d, FLSC cultured for 5 d; FLSC-21d, FLSC cultured for 21 d.

anti-c-Kit mAb (Figure 1A). Similar procedure using anti-biotin antibody coupled microbeads as described above was performed to sort c-Kit$^-$ cell population. Figure 2D shows the FACS analytical result of the negative fraction and it was revealed that more than 98% of the cells were c-Kit$^-$ cells.

Then we wondered whether these two separation procedures could be combined together and performed once to obtain the c-Kit$^-$(CD45/TER119)$^-$ cell population. Theoretically any unwanted cell that expressed either c-Kit or CD45/TER119 could be magnetically labeled and retained as a positive fraction on the separation column in a magnetic field and only the unlabeled cells, the c-Kit$^-$(CD45/TER119)$^-$ cells, could pass through. Our experiments indicated that this is feasible. After labeled with a cocktail of biotinylated antibodies (c-Kit, TER119 and CD45.2) and recognized by the anti-biotin microbeads, fetal liver cells were applied onto the separation column and the negative fraction was obtained. The purity of the c-Kit$^-$(CD45/TER119)$^-$ cells was 97.9% (Figure 3). The yield of this separation was about 6% of the total sorting cells. The cell viability was above 98% as measured by trypan blue dye exclusion.

Culture and identification of liver stem cells

To identify whether the immuno-magnetically separated c-Kit$^-$(CD45/TER119)$^-$ cells were liver stem cells, their capacity of colony-forming and multilineage differentiation was tested. After cultured *in vitro* for 4 d, obvious colonies (> 50 cells) were generated. Sorting for c-Kit$^-$(CD45/TER119)$^-$ cells (negative fraction) achieved more than 15-fold enrichment of colony-forming cells compared with total fetal liver cells, while the positive fraction of liver cell populations did not generate any colony (only three clones in 96 wells, probably due to c-Kit$^-$(CD45/TER119)$^-$ cell contaminants; data not shown). When we replated the cells from the formed clone onto new culture plates, several of these subcultured cells again formed colonies in 5 d (Figure 4).



Figure 4 *In vitro* subcolony formation from the sorted c-Kit$^-$(CD45/TER119)$^-$ cell. Culture of c-Kit$^-$(CD45/TER119)$^-$ cells was performed on laminin-coated 24-well plates for 4 d. Colonies were then digested and the cells from the formed clone were plated onto new culture plates and cultured for another 5 d. The picture shows a representative subcolony.

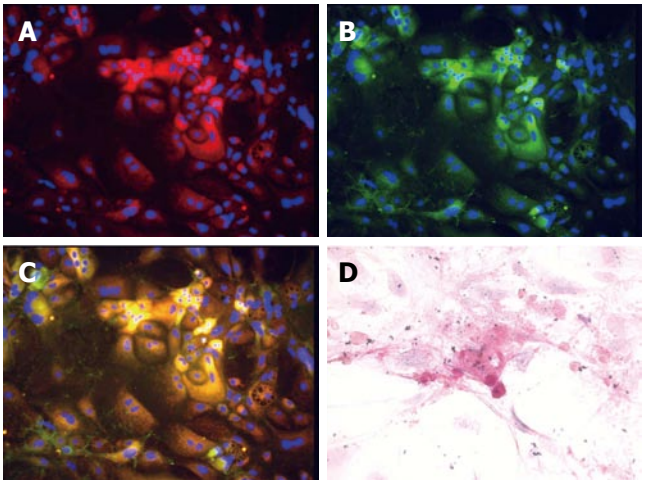


Figure 6 *In vitro* multilineage differentiation of ED 13 c-Kit$^-$(CD45/TER119)$^-$ fetal liver cells. The immunofluorescence pictures were obtained in 12-day cultures(**A** - **C**). Cells gave rise to albumin-positive hepatocytes (**A**, red) or cytokeratin 19-positive cholangiocytes (**B**, green). Also they gave rise to albumin and cytokeratin 19 double positive cells (yellow) and to double negative cells (**C**). The blue dots are DAPI nuclear signals. The original magnification was × 100. Intracellular glycogen was detected by periodic acid-Schiff staining(**D**). ED 13 c-Kit$^-$(CD45/TER119)$^-$ fetal liver cells gave rise to functionally mature hepatocytes, containing abundant glycogen stores, after 21 d of culture. The original magnification was × 400.

The hepatic differentiation of the cultured cells at d 5 and 21 was tested by their gene expression in RT-PCRs (*afp, alb* and *ck19* genes, Figure 5). AFP was expressed throughout the culture process. The expression of lineage markers *ALB* (for hepatocyte) and *CK19* (for cholangiocyte) was also detected. To verify the RT-PCR results, we stained colony-constituent cells at d 12 with

antibodies against albumin or cytokeratin 19. As shown in Figure 6, immunofluorescence revealed that most of cells expressed either albumin or cytokeratin 19 (Figures 6A and B). Two-color ALB/CK19 analysis revealed that ALB^+CK19^+ cells frequently accumulated in clusters, with hepatocytes and bile duct cells in peripheral segregated areas (Figure 6C). Periodic acid-Schiff staining showed that some cells had abundant glycogen stores at d 21 (Figure 6D), indicating functionally mature hepatocytes were generated. Taken together, these results suggest that the sorted $c\text{-}Kit^-(CD45/TER119)^-$ cells were capable of colony-forming and self-renewing and multilineage differentiation.

DISCUSSION

Separation of stem cells is typically done based on surface-marker expression. Previous studies showed that liver stem cells were enriched in the $c\text{-}Kit^-CD49f^+CD29^+CD45^-TER119^-$ fraction of mouse fetal liver cells by using flow cytometry and single cell-based assays[9] and were further enriched in the $c\text{-}Met^+CD49f^{+/low}c\text{-}Kit^-CD45^-TER119^-$ fraction[6]. Whereas oval cells, found in adult liver and marked for $c\text{-}Kit^+CD34^+Thy\text{-}1^+$, have been considered to be close descendants of these stem cells[6]. Similarly, about 10% of total ED 11 mouse fetal liver cells, marked for $c\text{-}Kit^{low}(CD45/TER119)^-$, showed in another study, was purified by FACS and considered to contain the earliest-described hepatic progenitors, also displaying features of liver stem cells[10]. Furthermore, a population of hepatoblasts in ED 14.5 fetal liver was isolated based on the expression of Dlk/Pref-1, a type I membrane protein. However, such Dlk^+ hepatoblasts represent more mature cells than $c\text{-}Met^+CD49f^{+/low}c\text{-}Kit^-CD45^-TER119^-$ cells[11]. Taken together the results of these studies, we concluded that liver stem cells could be enriched in the $c\text{-}Kit^-CD45^-TER119^-$ population. Our results presented in this report indicate that the $c\text{-}Kit^-CD45^-TER119^-$ population, purified by immuno-magnetic microbeads from ED 13 mouse fetal liver, was liver stem cells or enriched the liver stem cells significantly. As the markers for this population (c-kit and TER119/CD45) are all negative, it is easy to use magnetic microbeads to separate these cells at once.

As c-Met was considered to be the positive marker for liver stem cells, as described previously[6], it is also feasible to further separate the $c\text{-}Met^+$ population from the sorted $c\text{-}Kit^-(CD45/TER119)^-$ cells by positive selection using immuno-magnetic microbeads. Also since the liver stem cells have been pre-enriched in $c\text{-}Kit^-(CD45/TER119)^-$, using FACS to separate $c\text{-}Met^+$ cells could also be efficient.

There are several advantages of sorting liver stem cells using immuno-magnetic microbeads. First, this method is simple and inexpensive. Second, using this method, we can sort a total of 5×10^7 labeled fetal liver cells and obtain target cells with a high purity in 5 min, as compared with about 5 h by using an FACSAria cell sorter. As for proteomic analysis such as 2D-PAGE, 10^6-10^7 of cells with a high purity are needed, according to the dimension of the gel and staining method. Third, magnetically enriched populations help to reduce the prolonged exposure of liver stem cells to the high-pressure fluid environment in the flow cytometry, which might damage the biological activity of such a sensitive cell population. For biochemical and functional studies of stem cells, the total process relies on the viability of isolated cells which requires special attention during the cell isolation and purification procedures. In our experiments, the viability of the sorted liver stem cells by FACS was usually about 85%, as compared to above 98% by using immuno-magnetic microbeads. Fast and efficient acquisition of a large number of liver stem cells with high purity and viability is the basis for proteomic studies of liver stem cells.

In conclusion, the present study shows the application of immuno-magnetic microbeads for sorting and enrichment of liver stem cells from mouse fetal liver, by which the biological activity of the cells is kept. This method is fast, efficient, simple and not expensive.

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BASIC RESEARCH

Effect of recombinant human interleukin-11 on expressions of interleukin-11 receptor α-chain and glycoprotein 130 in intestinal epithelium cell line-6 after neutron irradiation

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Abstract

AIM: To explore the effect of recombinant human interleukin-11 (rhIL-11) on the expressions of interleukin-11 receptor α-chain (IL-11Rα) and an additional signal transducer glycoprotein 130 (gp130) in intestinal epithelium cell line-6 (IEC-6) after neutron irradiation.

METHODS: Cultured IEC-6 cells were exposed to 4.0Gy neutron and treated with 100 ng/mL rhIL-11 12 h prior to or immediately after irradiation. The apoptosis and necrosis rates and expressions of IL-11Rα and gp130 were observed by flow cytometry, immunohistochemistry, Western blot and image analysis.

RESULTS: The apoptosis rate of IEC-6 cells was increased by irradiation at 6 h ($P < 0.01$), IL-11 stimulation resulted in a decreased apoptosis rate in irradiated IEC-6 cells ($P < 0.05$). In normal control IEC-6 cells, intense immunoreactivity of IL-11Rα was located within the cell membrane and cytoplasm. The level of IL-11Rα expression significantly decreased at 6 h after irradiation ($P < 0.01$) and restored at 24 h after irradiation. In IEC-6 cells treated with both radiation and rhIL-11, the level of IL-11Rα expression was higher than that of irradiated cells ($P < 0.05$). When it came to gp130 protein, it was located in the cytoplasm of IEC-6 cells. After irradiation, we found a progressive decrease in the expression of gp130 protein ($P < 0.05$) in 48 h post-radiation, while in rhIL-11-stimulated cells, it came back to normal level at 24 h after irradiation and decreased at 48 h, but was still higher than that of only irradiated cells ($P < 0.05$).

CONCLUSION: rhIL-11 can protect IEC-6 cells from neutron irradiation. The protective effect of rhIL-11 might be connected with its ability to up-regulate the expressions of specific ligand-binding subunit IL-11Rα and signal-transducing subunit gp130.

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Key words: Neutron; IEC-6; Interleukin-11; IL-11Rα; Gp130

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INTRODUCTION

Intestine is highly sensitive to neutron irradiation. Once exposed to a given neutron irradiation dose, intestine is damaged severely and has become a severe limitation for patients on frequent radiotherapy for abdominal and pelvic malignancies.

Interleukin-11 (IL-11) is a member of a family of cytokines, including interleukin-6 (IL-6), leukemia inhibitory factor, oncostatin M, and ciliary neurotropic factor. IL-11-induced signaling is mediated by the formation of a hexameric receptor complex, composed of two molecules each of IL-11, IL-11Rα, and gp130[1]. Much attention has been paid to its actions on hematopoietic system[2,3]. Recently, IL-11 has been found to reduce intestinal epithelial injury due to a wide variety of causative incidents, including radiation and chemotherapy[4-11]. Yet the mechanisms of its irradiation protection are scarcely reported and no data on the action and mechanisms of IL-11 in the field of neutron irradiation are available.

In the present study, a model of normal rat intestinal epithelium cell line-6 (IEC-6) was established to observe the effect of recombinant interleukin-11 (rhIL-11) on the expressions of interleukin-11 receptor α-chain and gp130 in IEC-6 cells after neutron irradiation, which would be a base for further research of its effects on intestinal damage induced by irradiation and its mechanisms.

MATERIALS AND METHODS

Cell culture and rhIL-11 stimulation

IEC-6 cell line was kindly presented by Luo QL (Institute of Radiation Medicine, Academy of Military Medical Sciences, Beijing) and cultured in Dulbecco's modified Eagle' s medium (Sigma) supplemented with 10% fetal calf serum (FCS), 0.5% ciprofloxacin under standard tissue culture conditions (50 mL/L CO_2 at 37 ℃). The medium was changed every other day. Before treatment with rhIL-11 (Jiuyuan Genetics Co., Hangzhou), cells were incubated in medium containing 2.5% FCS for 24 h, and subsequently, 100 ng/mL rhIL-11 was administered 12 h prior to or immediately after irradiation. Thus cells in exponential growth period were randomly assigned to four groups: normal control cells, neutron irradiated cells, cells treated with rhIL-11 prior to irradiation (rhIL-11 pretreatment) and cells treated with rhIL-11 after irradiation (rhIL-11 post-treatment).

Neutron irradiation

Culture flasks containing IEC-6 cells were fixed on a tailor-made round plastic plate so that cells could rotate following the plate and absorb rays homogeneously when irradiated. The fission neutron reactor was provided by Institute of Nuclear and New Energy Technology, Tsinghua University. Cells were irradiated at a rate of 39.69cGy/min with a dose of 4.0Gy neutron ray absorbed.

Flow cytometry

Detection of apoptosis and necrosis was carried out according to the manufacturer's instructions (Biosea Biotechnology Co., Beijing). IEC-6 cells were detached from culture flasks with 2.5% trypsin (Sigma) to get monocyte suspension with a cell concentration of 5×10^5-1×10^6/mL, washed with ice-cold phosphate-buffered saline (PBS), centrifuged twice at 4000 r/min, and resuspended in 200 μL binding buffer. Then 10 μL Annexin-V-FITC and 5 μL propidium iodide (PI) were added for 15 min in the dark, and finally 300 μL binding buffer was added. The apoptosis and necrosis rates of the samples were analyzed by a FACS Calibr flow cytometer (BD Company, American).

Immunohistochemistry for IL-11Rα

IEC-6 cells were seeded onto glass tissue slides. Before staining, slides were fixed in a mixer with methanol and acetone (1:1) for 15 min at 4 ℃. After antigen repair with 0.25% trypsin, slides were incubated with rabbit anti- IL-11Rα mAb (1:80, Santa Cruz) for 1h at 37 ℃, then at 4 ℃ overnight, rinsed with PBS and incubated with biotinylated goat anti-rabbit IgG (1:200, Zhongshan Biotechnology Co., Beijing) for 50 min at 37 ℃. After rinsed three times with PBS, slides were treated with streptavidin-conjugated peroxidase (1:200, Zhongshan Biotechnology Co., Beijing) for 50 min at 37 ℃, incubated for 10-15 min in 3,3'-diaminobenzidine solution (Zhongshan Biotechnology Co., Beijing) at room temperature to allow color development and rinsed with distilled water to quench the reaction. Hematoxylin was used as a counterstain. PBS was used in stead of primary antibody as blank control.

Western blot for IL-11, IL-11Rα and gp130

Cells were washed sequentially twice with 1mL ice-cold PBS, centrifuged and resuspended in lysis buffer (1%SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCL, pH8.1, 1 mmol/L phenylmethylsulfonyl fluoride), sonicated on ice and centrifuged at 12 000 r/min for 15 min at 4 ℃. Protein concentration of the supernatant (protein fraction) was determined using a BCA protein assay reagent kit (Pierce). An aliquot of 25 μg of protein was mixed with an equivalent volume of 2× protein loading buffer containing 2-β-mercaptoethanol and boiled for 10 min before it was loaded onto SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked in PBS-T (0.1% Tween 20) containing 10% nonfat dry milk powder. Protein immunoblots were performed using specific antibodies to IL-11 (1:1000, Santa Cruz), IL-11Rα (1:800), gp130 (1:1000, Santa Cruz) and β-actin (1:1000, Santa Cruz). The membranes were further incubated with peroxidase-conjugated secondary antibodies (Zhongshan Biotechnology Co., Beijing), and protein bands were visualized using a commercial chemiluminescence detection kit (ECL Plus, Pierce) as described by the manufacturer. The prestained protein marker was from New England Biolabs Ltd, UK.

Image analysis and quantitation

The results of immunohistochemistry and Western blot were quantitated using a computer image analysis system (Beijing Aeronautic and Aerospace University) to assess the mean optical density (MOD) and the integral optical density (IOD) of the samples.

Statistical analysis

Data were expressed as mean ± SD. Results were analyzed by one-way ANOVA using SPSS 11.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Apoptosis and necrosis rates of IEC-6 cells

The apoptosis and necrosis rates of IEC-6 cells were increased significantly 6h after 4.0Gy neutron irradiation, while the apoptosis rate of IEC-6 cells treated with rhIL-11 pre- or post-radiation was much lower than that of only irradiated cells, but the necrosis rate remained unchanged (Figure 1).

Expression of IL-11 in IEC-6 cells

IL-11 expression was found in the cytoplasm of normal IEC-6 cells. The expression of IL-11 was significantly reduced 6 h after 4.0Gy neutron irradiation and returned to normal level 24 h after 4.0Gy neutron irradiation (Figure 2). The quantitative results are shown in Table 1.

Expression of IL-11Rα in IEC-6 cells

Immunohistochemical staining analysis of IL-11Rα expression: In normal control IEC-6 cells, intense immunoreactivity of IL-11Rα was located within the cellular membrane and cytoplasm. Six hours after 4.0Gy neutron irradiation, only a very slight immunoreactivity was found

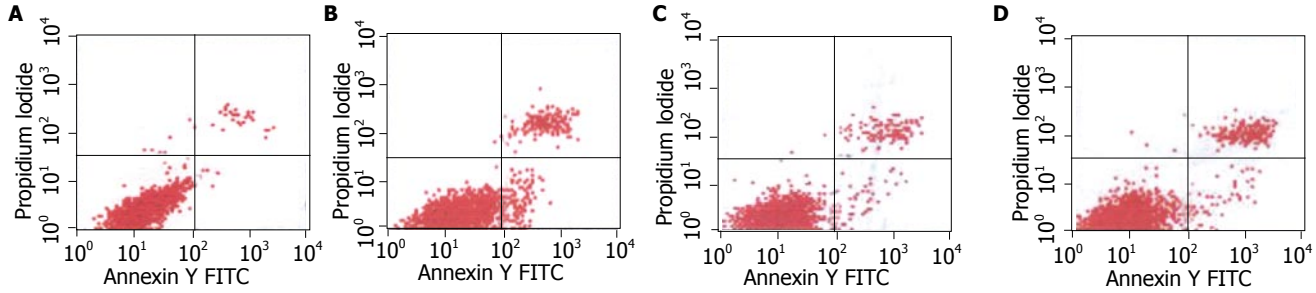


Figure 1 Effects of rhIL-11 treatment on apoptosis and necrosis rates of IEC-6 cells 6 h after 4.0Gy neutron irradiation. **A:** The apoptosis and necrosis rates of normal IEC-6 cells were 0.79% and 1.99%, respectively; **B:** In 4.0Gy neutron-irradiated IEC-6 cells, the apoptosis and necrosis rates were 5.58% and 9.04%, respectively; **C:** In IEC-6 cells treated with rhIL-11 before neutron irradiation, the apoptosis rate was 1.91%, lower than that without rhIL-11 stimulation; **D:** In IL-11 post-treatment IEC-6 cells, the apoptosis rate was 1.86%, lower than that without rhIL-11 stimulation. But in both rhIL-11-treated IEC-6 cells, the necrosis rate was not decreased obviously.

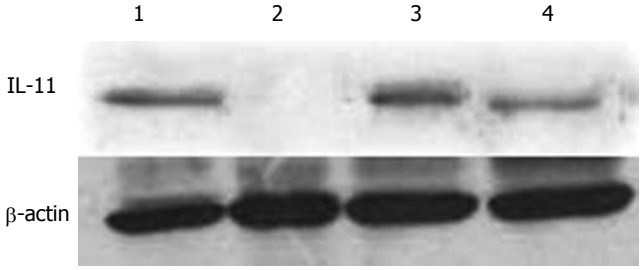


Figure 2 Effect of 4.0Gy neutron irradiation on IL-11 expression of IEC-6 cells. Lane 1: normal control group; lanes 2-4: 6, 24 and 48 h after 4.0Gy neutron irradiation, respectively.

Table 1 Quantitative results of IL-11 expression in IEC-6 cells after 4.0Gy neutron irradiation (mean ± SD)

Time after irradiation (h)	MOD ($\times 10^{-2}$)	IOD ($\times 10^{-1}$)
0	17.33 ± 1.16	17.30 ± 1.04
6	6.33 ± 0. 58 [a]	1.30 ± 0.17 [a]
24	24.67 ± 3.22	27.30 ± 2.59
48	14.33 ± 0. 58	13.33 ± 0.50

n = 3. [a]$P < 0.05$ *vs* normal control group.

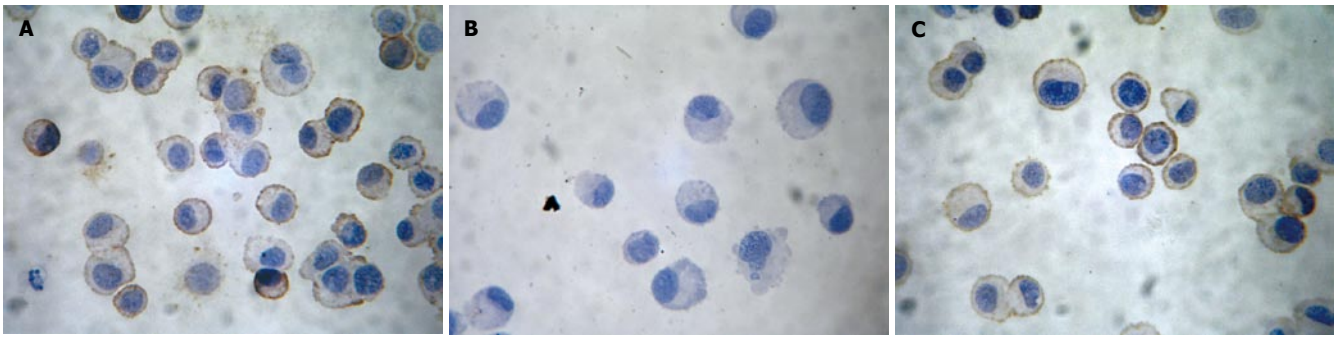


Figure 3 Immunohistochemichal staining of IL-11Rα (400×) located in cytomembrane and cytoplasm of IEC-6 cells. **A:** IL-11Rα was strongly positive in normal IEC-6 cells; **B:** Six hours after 4.0Gy neutron irradiation, dramatic decline was found in IL-11Rα expression; **C:** No obvious decline was observed in IEC-6 cells treated with 100 ng/mL rhIL-11 after 4.0Gy neutron irradiation.

but became normal 24 h after irradiation. In IEC-6 cells treated with both radiation and rhIL-11, there was no decrease in the expression of IL-11Rα (Figure 3). The quantitative results are listed in Table 2.

Western blot analysis of IL-11Rα expression: The results were mainly in accord with those of immunohistochemistry (Figure 4). The quantitative results are listed in Table 3.

Expression of gp130 in IEC-6 cells

gp130 protein was observed in normal IEC-6 cells. After irradiation, gp130 expression was progressively decreased in within 48 h. In rhIL-11-pretreated cells, despite a decrease 6 h post-irradiation, gp130 expression became normal at 24 h and decreased a little at 48 h. Besides, in IEC-6 cells treated with rhIL-11administration after irradiation, gp expression was decreased slightly 48 h after irradiation and no obvious changes were found 6 and 24 h after irradiation. gp130 protein expression in rhIL-11-stimulated cells 24 h and 48 h after irradiation was higher than that of the irradiated cells (Figure 5). The quantitative results are shown in Table 4.

DISCUSSION

IL-11 is a functionally pleiotropic cytokine originated from the primate PU34 bone marrow fibroblast cell line[12]. Though initially known as a hematopoietic growth factor, IL-11 is an important mediator in systems other than the hematopoietic system. Studies revealed that IL-11 plays an important role in intestinal epithelial injury and repair[4-11], immunomodulation[13-14], neurogenesis[15-16], osteoclasto-

Table 2 Quantitative results of IL-11Rα expression in IEC-6 cells after 4.0Gy neutron irradiation (IHC) (mean ± SD)

Time after irradiation (h)	MOD (×10^{-2})			IOD (×10^{-1})		
	Normal	Neutron-radiated	rhIL-11 post-treatment	normal	Neutron- radiated	rhIL-11 post-treatment
6	8.60 ± 2.07	3.40 ± 0. 97[b]	10.50 ± 1.08[a]	24.25 ± 10.33	1.32 ± 1.04[b]	42.76 ± 18.27[a]
24	-	10.38 ± 1.23	17.80 ± 2.53[b a]	-	37.95 ± 12.54	100.88 ± 47.57[a]

n = 3. [b]P < 0.01 *vs* normal control group; [a]P < 0.05 *vs* neutron irradiation group.

Table 3 Quantitative results of IL-11Rα expression in IEC-6 cells after 4.0Gy neutron irradiation (mean ± SD)

Time after irradiation (h)	MOD (×10^{-2})			IOD (×10^{-1})		
	normal	Neutron-radiated	rhIL-11 post-treatment	normal	Neutron-radiated	rhIL-11 post-treatment
6	5.30 ± 0.10	3.07 ± 0. 31[a]	4.23 ± 0.12[a c]	82.13 ± 3.51	8.10 ± 2.45[b]	40.27 ± 2.64[b c]
24	-	4.97 ± 0.15	5.33 ± 0.14[c]	-	44.80 ± 1.21[b]	70.03 ± 2.2[c]

n = 3. [a]P < 0.05, [b]P < 0.01 *vs* normal control group; [c]P < 0.05, [d]P < 0.01 *vs* neutron irradiation group.

Table 4 Quantitative results of gp130 expression in IEC-6 cells after 4.0Gy neutron irradiation (mean ± SD)

Time after irradiation (h)	MOD (×10^{-1})				IOD (×10^{-1})			
	Normal	Neutron-radiated	rhIL-11 pretreatment	rhIL-11 post-treatment	Normal	Neutron-radiated	rhIL-11 pretreatment	rhIL-11 post-reatment
6	4.90 ± 0.44	3.70 ± 0.17	3.83 ± 0.06	5.37 ± 0.58[a]	51.07 ± 16.75	26.27 ± 10.25[b]	35.60 ± 4.03	68.60 ± 5.19[c]
24	-	2.70 ± 0.15[a]	5.53 ± 0.08[c]	5.50 ± 0.11[c]	-	19.13 ± 5.14[b]	89.79 ± 5.12[a d]	75.87 ± 0.68[c]
48	-	1.61 ± 0.10[a]	4.47 ± 0.15[d]	2.63 ± 0.15[c]	-	7.46 ± 0.50[b]	41.33 ± 13.55[a c]	18.70 ± 0.10[b c]

n = 3. [a]P < 0.05, [b]P < 0.01 *vs* normal control group; [c]P < 0.05, [d]P < 0.01 *vs* neutron irradiation group.

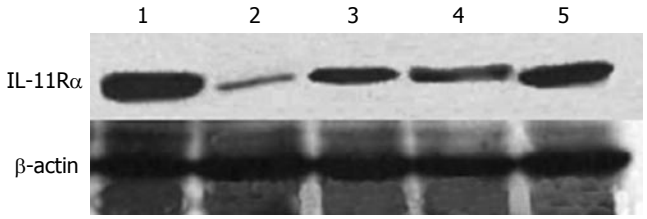


Figure 4 IL-11Rα and β-actin expressions in IEC-6 cells by Western blot. Lane 1: normal control group; lanes 2-3: 6 and 24 h after 4.0Gy neutron irradiation; lanes 4-5: 6 and 24 h of rhIL-11 treatment after irradiation.

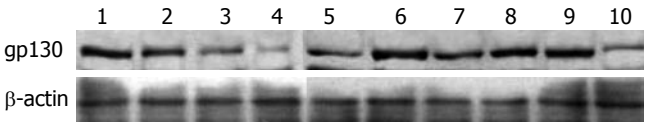


Figure 5 gp130 protein and β-actin expressions in IEC-6 cells by Western blot. Lane 1: normal control group; lanes 2–4: 6, 24 and 48 h after 4.0Gy neutron irradiation respectively; lanes 5-7: 6, 24 and 48 h of rhIL-11 pretreatment group; lanes 8-10: 6, 24 and 48 h of rhIL-11 treatment after irradiation.

genesis[17], general homeostasis of the skin, *etc*[18-19]. Present researches involving intestinal injury and repair on instance intestinal damage induced by chemotherapy and radiation[6, 8-11], inflammatory bowel disease[20], bowel ischemical reperfusion injury[4], short bowel syndrome[7], etc, have proved that IL-11 can prevent injury and accelerate recovery.

Previous studies indicated that IL-11 could exert a potent effect on the recovery of the small intestinal mucosa injury in mice treated with radiation and/or chemotherapy by its combined effects on proliferation and apoptosis of crypt cells[9]. However, the effects of IL-11 are not exclusively stimulatory. *In vitro* studies showed that IL-11 directly interacts with intestinal epithelial cells and reversibly inhibits proliferation of the intestinal crypt stem cell lines (IEC-6 and IEC-18)[21]. It seems contradictory that IL-11 can not only stimulate but inhibit proliferation. Possibly it is because that IL-11 exerts different effects on damaged and undamaged cell clusters. In respect to the undamaged cells, it inhibits proliferation (as reduction of cellular proliferation can be cytoprotective, pretreatment with IL-11 may reduce radiation sensitivity before exposed to rays) and promotes proliferation of damaged cells (enhancing recovery after radiation). Other studies also found that IL-11 pretreatment induces intestinal epithelial cell growth arrest through effects on retinoblastoma protein phosphorylation and delays entry into S phase of the cell cycle[22]. Transient cell cycle arrest is a possible mechanism by which rhIL-11 may protect intestinal epithelial cells from damage induced by chemotherapy or radiation therapy. In this study, pretreatment with rhIL-11 prior to neutron irradiation led to a decrease in apoptosis rate, which may be due to its direct interaction with IEC-6 cells and prolongation of G_1-S phase consequently reducing the radiosensitivity of IEC-6 cells. Data also have shown that

IL-11 is able to protect intestinal crypt stem cells from impairment of their reproductive capacity by radiation[10]. IL-11-administeration after radiation can cause a greater number of crypt cells to survive a given radiation dose[10]. In this study, a decrease in apoptosis rate was found in cells post-treated with IL-11 after irradiation, which may be due to the fact that rhIL-11 stimulation promotes the survived IEC-6 cells to reproduce, leading to the decrease of apoptosis rate. In addition, 4.0Gy neutron irradiation resulted in a significant reduction of IL-11 expression in IEC-6 cells 6 h after irradiation. Simultaneously, flow cytometry assay showed an increased apoptosis rate, indicating that IL-11 can protect IEC-6 cells from neutron irradiation.

It was reported that IL-11 responsiveness is restricted to cells expressing both the IL-11Rα subunit and the transmembrane signaling receptor gp130 on their surface[23]. The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is an important signaling system activated by the IL-11Rα. It was reported that IL-11 stimulation results in decreased activation of caspase-9 and reduced induction of apoptosis in cultured colonic epithelial cells (CEC)[23]. In this study both IL-11Rα and gp130 expressions were decreased in irradiated cells without rhIL-11 treatment 6 h after irradiation (the course of injury). However, in rhIL-11-treated cells, receptor expression level was higher and apoptosis rate was lower than those in irradiated cells without rhIL-11 treatment, indicating that increased IL-11 receptor complex expression induced by rhIL-11 stimulation is involved in the injury and repair of IEC-6 cells caused by neutron irradiation. In irradiated cells, the decreased IL-11 receptor expression is unbeneficial for IL-11 to exert radioprotection effect, thus increasing the apoptosis rate. However in rhIL-11-treated cells, rhIL-11 treatment not only upregulates the expression of IL-11 receptor but also is beneficial to exert its actions, thus reducing the apoptosis rate induced by irradiation. Alterations of receptor expression reflect changes in activation of corresponding signaling pathway. The alteration of IL-11 downstream signal pathway in IEC-6 cells treated with neutron irradiation and rhIL-11 is now under investigation.

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BASIC RESEARCH

Effect of early administration of exogenous basic fibroblast growth factor on acute edematous pancreatitis in rats

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Abstract

AIM: To observe the therapeutic effect of early administration of exogenous Basic fibroblast growth factor (bFGF) on acute edematous pancreatitis (AEP) in rats.

METHODS: Thirty male Sprague-Dawley rats were randomly divided into three (n = 10): normal control group (group I), AEP group (group II) and AEP with bFGF treatment group (group III). AEP was induced by subcutaneous injection of cerulein (5.5 μg/kg and 7.5 μg/kg) at 1 h interval into rats of groups II and III. Three hours after induction of AEP, 100 μg/kg bFGF was administrated intraperitoneally for 1h to group III rats. For test of DNA synthesis in acinar cells, *5-bromo-2'-deoxyuridine* (*BrdU*) labeling solution was intraperitoneally injected into the rats of groups II and III 24 h after bFGF treatment. The changes in serum amylase, lipase, pancreatic tissue wet/dry ratio were detected.

RESULTS: In bFGF treatment group, there was a significant decrease in the volume of serum amylase, lipase and the pancreatic wet/dry weight ratio(1383.0 ± 94.6 U/L, 194.0 ± 43.6 U/L, 4.32 ± 0.32) compared to AEP group (3464 ± 223.7 U/L, 456 ±68.7 U/L, 6.89 ± 0.47) (P < 0.01), and no significant difference was found between bFGF treatment and control group (1289 ± 94.0 U/L, 171 ± 23.4 U/L, 4.12 ± 0.26, P > 0.05). The inflammatory changes such as interstitial edema, polymorphonuclear neutrophils (PMNs) and vacuolization were significantly ameliorated compared to AEP group (P < 0.01). A small number of BrdU-labeled nuclei were observed in acinar cells of AEP rats (1.8 ± 0.3 nuclei/microscopic field, n = 10) while diffuse BrdU-labeled nuclei were found in bFGF-treated rats (18.9 ± 1.4 nuclei/microscopic field, n = 10) (P < 0.01). Immunohistochemical study showed increased DNA synthesis in pancreatic acinar cells.

CONCLUSION: Early administration of exogenous bFGF has significant therapeutic effect on cerulein-induced acute edematous pancreatitis in rats. Its mechanism is related to the amelioration of inflammation and facilitation of pancreatic regeneration.

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Key words: Basic fibroblast growth factor; Acute edematous pancreatitis; Cerulein

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INTRODUCTION

Basic fibroblast growth factor (bFGF) is one of the mitogens that facilitate cellular proliferation, maturation and regeneration[1]. Hoshi *et al*[2] reported that bFGF exerts direct trophic effects on rat pancreatic acinar cells *in vitro*. Some studies[3-6] showed that FGF is involved in the process of acute pancreatic exocrine regeneration during recovery of acute pancreatitis. Acute edematous pancreatitis (AEP) rats were selected as the animal model to investige the therapeutic effect of exogenous bFGF on the early acute pancreatitis (AP) and its mechanism.

MATERIALS AND METHODS

Animal model

Thirty male healthy Sprague-Dawley rats weighing 400-450 g were purchased from the Animal Center, Academy of Zhejiang Medical Sciences. All rats were randomly divided into three groups (n = 10), with 10 rats each group. Group I (normal control group), group II (AEP group), and group III (AEP with bFGF treatment group). AEP was induced in rats of goups II and III by injection of 5.5 μg/kg and 7.5 μg/kg of caerulein (Sigma Chemical Co., USA) subcutaneously at 0 and 1 h after the beginning of experiment respectively[7, 8], while the rats of the control group were subcutaneously injected with normal saline solution.

bFGF treatment and 5-bromo-2'-deoxyuridine (BrdU) label

All animals were anesthetized with 3% thiopental sodium

(0.3 μL/g). In group III bFGF (100 μg/kg) was administered by contiunuous intraperitoneal infusion of 10 μg/mL bFGF solution (Essex Co., China) for 1 h, while normal saline solution was administered to all other rats in groups I and II as the contrast. Twenty-four hours after treatment, 200 μg/mL BrdU-labeling solution (10 mL/kg) was intraperitoneally injected into the rats of groups II and III.

Blood and tissue sample collection

Two hours after BrdU-labeling solution injection, venous blood was taken from all rats in each experimental group to obtain blood samples. Then all rats were killed and pancreatic tissue was divided into 3 portions from each rat and quickly removed.

Histological examination

A portion of the pancreas was fixed overnight in 10% neutral formaldehyde solution and embedded in paraffin. Tissue slices were subjected to hematoxylin and eosin staining and histologic study under light microscope. Slides were coded and examined blindly by the pathologist for the grading of histologic alterations. Histological changes were evaluated according to Kyogoku *et al*[9]. Interstitial edema was scored as 0 = absent, 1 = expanded interlobular septa, 2 = expanded intralobular septa, 3= separated individual acini. Polymorphonuclear neutrophil (PMN) infiltration was scored as 0 = absent, 1=less than 20 PMNs per IPF, 2 =20 PMNs-50 PMNs per IPF and 3 = more than 50 PMNs per IPF. The grading of vacuolization was based on the percentage of acinar cells with cytoplasmic vacuoles per IPF: 0 = absent, 1 = less than 20%, 2 = 20%-50%, 3 = more than 50%. Hemorrhagic and parenchymal necrosis changes were absent and therefore not scored.

Immunohistochemistry examination (S-P method)

DNA synthesis of pancreatic acinar cells was determined as the uptake of Brdu in the cells. Another portion of the pancreas was fixed in 10% formalin, embedded in paraffin, and sectioned at 3 mm. After dewaxing, the tissue was then incubated with the monoclonal antibodies against BrdU for 1 h at room temperature in a humidified chamber. The tissue was washed three times with 0.01 mol/L phosphate-buffered saline (PBS) and incubated in peroxidase-conjugated anti-mouse immunoglobulin G (IgG) for 30 min at room temperature. The tissue was again washed three times with 0.01 mol/L PBS, and immunoreactivity of BrdU was detected using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) with nickel chloride as a chromogen. DAB (10 mg) was dissolved in 0.05 mol/L phosphate buffer (30 mL), and three drops of nickel chloride (3%) and hydrogen peroxide (3%) solution were also added. The DAB solution was applied to the tissue for 5 min at room temperature. The BrdU-labeled nuclei were stained purplish blue.

Statistical analysis

All data were expressed as mean ± SD. Histologic scores were compared using the Mann-Whitney test, while statistical differences of serum amylase were performed using paired Student's *t* test. $P < 0.05$ was considered statistically significant.

Table 1 Changes in serum amylase, lipase and pancreatic wet/dry weight ratio (n = 10) (mean ± SD)

Groups	Serum amylase (U/L)	Serum lipase (U/L)	Pancreatic wet/dry weight ratio
Group I	1289 ± 94.0	171 ± 23.4	4.12 ± 0.26
Group II	3464 ± 223.7[b]	456 ± 68.7[b]	6.89 ± 0.47[b]
Group III	1383 ± 94.6[ad]	194 ± 43.6[ad]	4.32 ± 0.32[ad]

[a]$P > 0.05$, [b]$P < 0.01$ *vs* group I; [d]$P < 0.01$ *vs* group II.

Table 2 Pathological lesion score for pancreas of each group (n = 10) (mean ± SD)

Groups	Edema	PMN infiltration	Vacuolization
Group I	0	0	0
Group II	2.40 ± 1.07[b]	2.50 ± 1.08[b]	2.40 ± 0.96[b]
Group III	0.70 ± 0.67[ad]	0.60 ± 0.59[ad]	0.70 ± 0.62[ad]

[b]$P < 0.01$, [a]$P < 0.05$ *vs* group I; [d]$P < 0.01$ *vs* group II.

RESULTS

Serum amylase, lipase and pancreatic tissue wet/dry weight ratio

When SEP model was established and bFGF treatment was taken after 24 h, serum amylase and lipase activity as well as pancreatic tissue wet/dry weight ratio of group III (bFGF treatment group) (1383.0 ± 94.6 U/L, 194.0 ± 43.6 U/L, 4.32 ± 0.32) were significantly lower than those of group II (AEP group) (3464 ± 223.7 U/L、456 ± 68.7 U/L and 6.89 ± 0.47) ($P < 0.01$), with no significant difference compared with group I (normal control group) (1289 ± 94.0 U/L、171 ±23.4 U/L and 4.12 ± 0.26)($P > 0.05$). There was a significant difference between group I and II ($P < 0.01$) (Table 1).

Histological staining

In the histological examination of pancreatic tissue stained with HE, pancreatitis changes such as angioectasia and inflammatory cell infiltration, tissue edema and vacuolization of acinar cells in bFGF treatment group(group III) were remarkably reduced compared with AEP group (group II) (Table 2 and Figures 1-3).

Immunohistochemical analysis

A small number of BrdU-labeled nuclei were observed in acinar cells of AEP rats (group II) (1.8 ± 0.3 nuclei/microscopic field, n = 10) (Figure 4A). The BrdU-labeled nuclei were diffusely distributed in bFGF treated rats (group III) (18.9 ± 1.4 nuclei/microscopic field, n = 10) ($P < 0.01$) (Figures 4B).

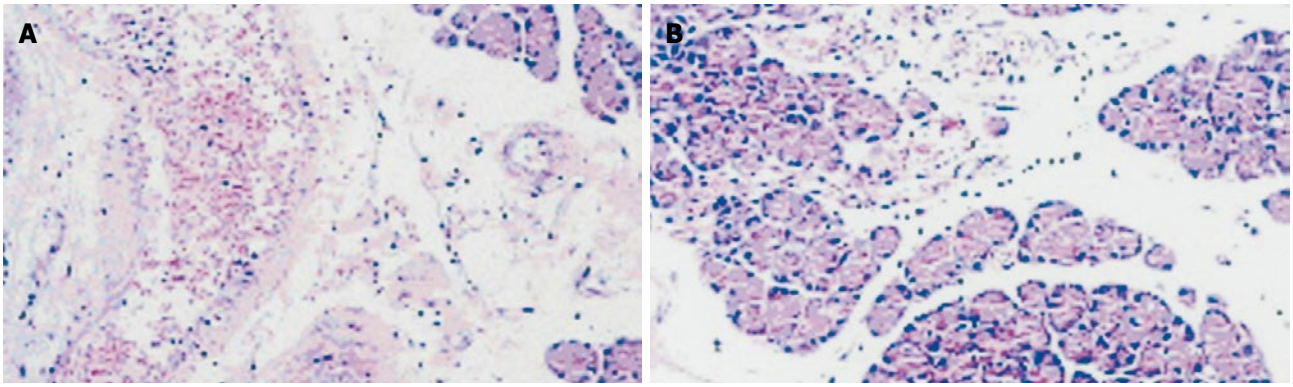


Figure 1 Marked angioectasia and inflammatory cell infiltration of acinar cells (**A**) and marked interstitia edema and inflammatory cell infiltration of acinar cells (**B**) observed in AEP rats under light microscope (×100).

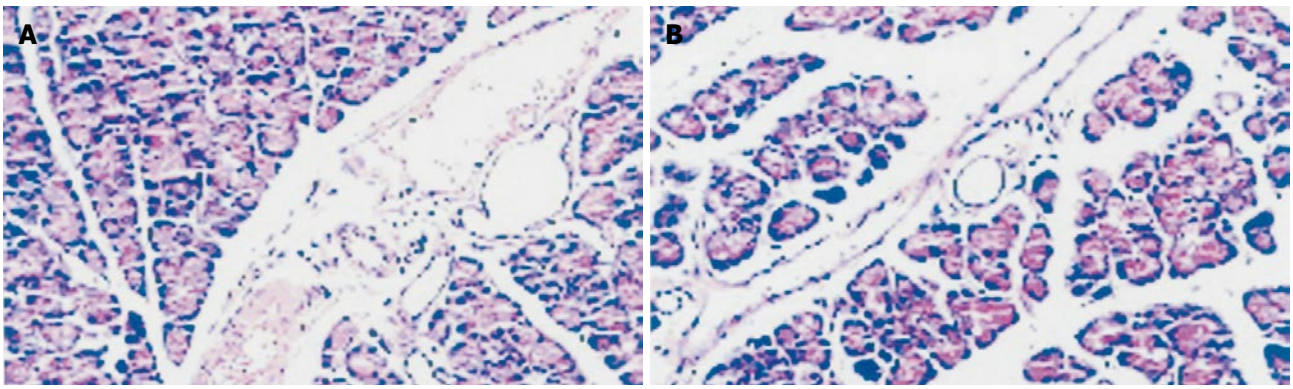


Figure 2 Significant amelioration of angioectasia and inflammatory acinar cell infiltration (**A**) and mild interstitia edema and angioectasia (**B**) in rats after bFGF treatment under light microscope (×100).

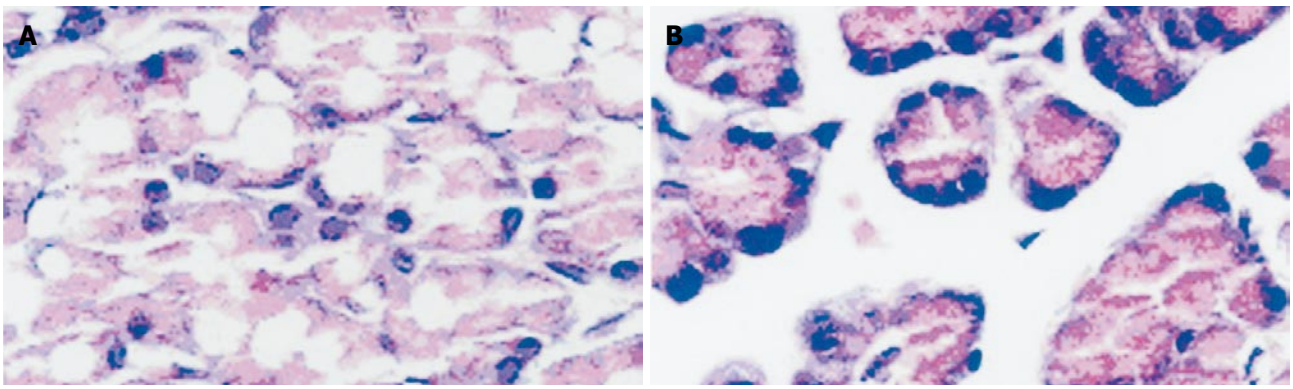


Figure 3 Marked tissue edema and vacuolization of acinar cells group II (**A**) compared to group III (**B**) after bFGF treatment under light microscope (×400).

DISCUSSION

Some factors can repair the injured tissues or cells[10-14] and can be used to treat acute pancreatitis. Recent studies indicate that various growth factors such as bFGF[15-17], TGF-β1[18,19], HGF[6, 20], EGF[18, 21-24] and CTGF[25] are involved in pancreatic repair and tissue remolding in humans and rats with AP. It was reported that ischemia/reperfusion (I/R) injury of intestine and other internal organs reduces the expression of endogenous basic fibroblast growth factor (bFGF) [26-29], and intravenous administration of exogenous bFGF could induce the expression of endogenous bFGF and improve the physiological function of some internal organs after I/R injury[30-33]. So there may be a bright future to accelerate the "positive repair" of internal organs' I/R injuries by using growth factors based on all these results.

The present study used acute edematous pancreatitis rats as a model to observe the therapeutic effect of exogenous bFGF on acute edematous pancreatitis in rats. Early treatment with exogenous bFGF drastically ameliorated all the pathological changes both in histology and in serum enzymes, and showed significant therapeutic effect on ce-

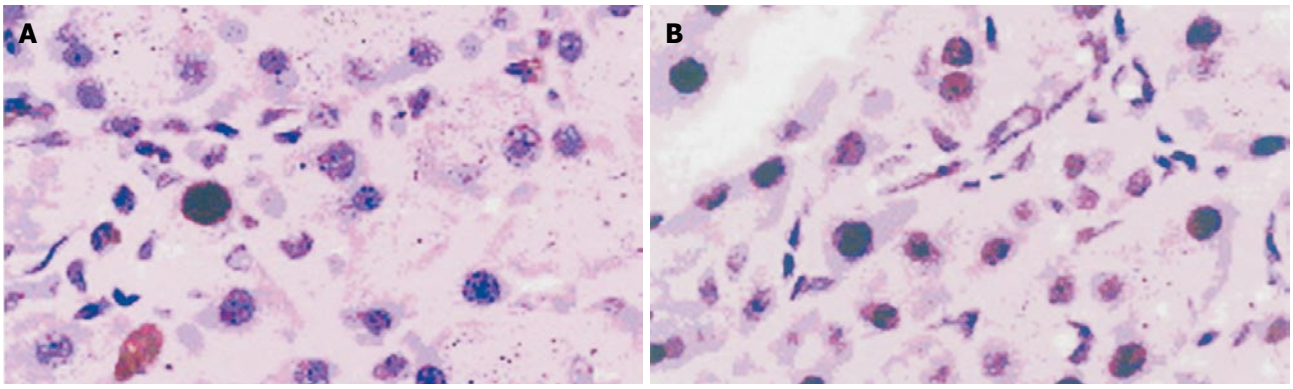


Figure 4 BrdU labeled nuclei of acinar cells in group II (**A**) and group III (**B**) after bFGF treatment (S-P method, ×400).

rulein-induced acute edematous pancreatitis in rats. Immunohistochemical examination showed that DNA synthesis was increased in pancreatic acinar cells of bFGF-treated rats, suggesting that bFGF treatment can promote pancreatic cell regeneration and accelerate rebuilding the integration of injured pancreatic tissue. The beneficial effects of bFGF appear to depend, at least in part, on amelioration of pancreatic inflammation and increase of pancreatic cell growth. Results of the present study have proved that early usage of exogenous bFGF can prevent the development of acute pancreatitis, and may play an important role in limiting its progression to sever pancreatitis.

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RAPID COMMUNICATION

Hepatitis B virus x gene and cyanobacterial toxins promote aflatoxin B_1-induced hepatotumorigenesis in mice

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Abstract

AIM: To assess the combinative role of aflatoxin B_1 (AFB_1), cyanobacterial toxins (cyanotoxins), and hepatitis B virus (HBV) x gene in hepatotumorigenicity.

METHODS: One-week-old animals carrying HBV x gene and their wild-type littermates were intraperitoneally (ip) injected with either single-dose AFB_1 [6 mg/kg body weight (bw)], repeated-dose cyanotoxins (microcystin-LR or nodularin, 10 μg/kg bw once a week for 15 wk), DMSO (vehicle control) alone, or AFB_1 followed by cyanotoxins a week later, and were sacrificed at 24 and 52 wk post-treatment.

RESULTS: AFB_1 induced liver tumors in 13 of 29 (44.8%) transgenic mice at 52 wk post-treatment, significantly more frequent than in wild-type mice (13.3%). This significant difference was not shown in the 24-wk study. Compared with AFB_1 exposure alone, MC-LR and nodularin yielded approximately 3-fold and 6-fold increases in the incidence of AFB_1-induced liver tumors in wild-type animals at 24 wk, respectively. HBV x gene did not further elevate the risk associated with co-exposure to AFB_1 and cyanotoxins. With the exception of an MC-LR-dosed wild-type mouse, no liver tumor was observed in mice treated with cyanotoxins alone at 24 wk. Neither DMSO-treated transgenic mice nor their wild-type littermates had pathologic alterations relevant to hepatotumorigenesis in even up to 52 wk.

CONCLUSION: HBV x gene and nodularin promote the development of AFB_1-induced liver tumors. Co-exposure to AFB_1 and MC-LR tends to elevate the risk of liver tumors at 24 wk relative to exposure to one of them. The combinative effect of AFB_1, cyanotoxins and HBVx on hepatotumorigenesis is weak at 24 wk.

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Key words: Aflatoxins; Cyanobacteria; Hepatitis B virus; Liver neoplasms; Transgenic mice

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer mortality worldwide[1]. In the People's Republic of China, HCC is the second leading cause of cancer mortality with 28 463 deaths in 1999[2]. Epidemiological studies revealed that multiple environmental factors were associated with HCC in some high-risk areas. Chronic hepatitis B virus (HBV) infection and dietary aflatoxin exposure are two major etiological risk factors for HCC, which has been studied in the past two decades[3]. For individuals chronically infected with HBV, concurrent exposure to dietary aflatoxins increases the risk of HCC by at least three-fold[4]. Continuous hepatocyte death and regeneration caused by host immune response to viral antigens contributes to the pathogenesis of chronic HBV infection[5]. The HBV x (HBVx) gene, one of the four genes in the HBV genome, encodes a polypeptide of 154 amino acids and is highly conserved among all mammalian hepadnaviruses. Interestingly, in the absence of HBV x gene, duck hepatitis B virus (DHBV) infection did not yield HCC, but the frequent occurrence of HCC was caused by infections of woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) with the presence of HBV x gene[6]. Compared to other regions of the genome, the viral DNA sequences encoding HBV X and/or truncated envelope PreS2/S proteins were more frequently detected in the primary tumors and tumor-derived cell lines. Although HBVx does not bind to DNA directly, it is capable of trans-activating cellular

genes and is considered to be a tumor promoter during liver carcinogenesis[7]. Some HBV x gene transgenic animals showed an increased susceptibility to chemical hepatocarcinogens[8,9]. Studies using transgenic mouse lines lend further support for the epidemiological finding that chronic HBV infection acts synergistically with aflatoxins in hepatocelluar carcinogenesis[10,11].

However, other risk factors, such as chronic exposure to cyanobacterial toxins (cyanotoxins) through drinking water, were relatively less studied or even overlooked. The mortality rate of HCC was closely associated with the source of drinking water in southern China at high risk of HCC[12]. Wide contamination of cyanotoxins in drinking water sources in these endemic areas suggests that the high incidence of HCC may be associated, in part, with chronic exposure to cyanotoxins via drinking water[13]. Cyanobacteria produce a wide range of secondary metabolites that are hazardous to humans, livestock, and wildlife[14]. Several bloom-forming cyanobacterial genera, including *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc*, are capable of producing the cyclic heptapeptide toxins, microcystins and *Nodularia*, the latter further metabolized to the cyclic pentapeptide nodularin. These toxins have a number of common structural features, in particular, the unique β-C20 amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. The most widely studied microcystins is microcystin-LR (MC-LR) with an LD_{50} between 32.5 μg/kg body weight (bw) and 10.9 mg/kg bw in rodents by intraperitoneal (ip) injection, oral, and aerosol inhalation routes[15-17]. The direct cause of death in toxin-treated rodents was hemorrhagic shock and liver necrosis. MC-LR is a potent tumor promoter as well[18]. A two-stage carcinogenesis test showed that MC-LR modulated aflatoxin B_1-induced hepatocarcinogenicity. In rats given AFB_1 as an initiator, the subsequent treatment with MC-LR resulted in a synergistic increase in the development of glutathione S-transferase placental form (GST-P)-positive liver cell foci[19]. The hepatotoxicity of MC-LR is, at least in part, explained by its ability to inhibit protein phosphatases 1 and 2A and consequently induces hyperphosphorylation of various proteins, including tumor suppressor gene products, retinoblastoma (Rb) and p53[20]. Nodularin injures mammalian and fish livers in the same manner as microcystins; it also inhibits protein phosphatases and acts as a tumor promoter[21]. The result of a two-stage carcinogenesis experiment indicated that nodularin has the potential to stimulate growth of the initiated liver cells more strongly than does microcystin-LR and, additionally, an initiating capacity similar to diethylnitrosamine (DEN)[22].

Although epidemiological studies conducted in high-risk areas in China have implicated the synergistic effect of chronic HBV infection, dietary AFB_1 exposure and chronic exposure to cyanotoxin-contaminated drinking water on the development of HCC, no experimental design has been made so far to study comprehensively the combinative effect of these three environmental risk factors on the development of liver tumors in animals. In this study, we attempted to use the established HBV x gene transgenic mouse model to assess the combinative role of chemical and viral infection in hepatotumorigenicity.

MATERIALS AND METHODS

Reagents

Aflatoxin B_1 (AFB_1) was purchased from Sigma Chemical Co. (St. Louis, MO). MC-LR (> 90% purity in HPLC), generously provided by Dr. Li-Rong Song, Department of Phycology, Institute of Hydrobiology, Chinese Academy of Sciences, was dissolved in saline to a concentration of 0.5 mg/mL. Nodularin (> 95.0% purity in HPLC), kindly provided by Dr. Wayne W. Carmichael, Wright State University, was dissolved in dimethylsulfoxide (DMSO, Burdick & Jackson, Muskegon, MI) to a concentration of 5 mg/mL. All stock solutions were kept at -20 ℃ until use.

Hepatitis B virus x gene transgenic mice

The development of the HBV x gene transgenic mice used in this study has been reported elsewhere[23]. Briefly, a recombinant retroviral expression vector plasmid pSHDX42 containing a 0.59 kilobase (kb) HBV subtype *adr* DNA fragment which spans nucleotide position 1 248 to 1 841 in the viral genome was constructed. A retroviral enhancer LTR, SV40 polyadenylation sequence, and a universal terminator from the expression vector pLXSHD were also inserted into pSHDX42 to initiate and terminate the transcription of the x gene. The DNA segment in the vector pSHDX42 contained the entire coding region of the x gene (map positions 1376 to 1840) and was released from pSHDX42 by *Bam*H Π/*Sph* I digestion. The purified DNA was microinjected into the pronuclei of fertilized eggs of C57BL/6J mice purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences. The DNA-injected eggs were transferred into the oviduct of pseudopregnant female ICR mice purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences. Genomic DNA and total RNA were extracted from F1 generation tail and liver tissues. Transgenic mice were identified by the methods of nested-primer PCR and Southern blotting of tail genomic DNA as well as RT-PCR of liver mRNA and immunohistochemical staining of HBV X protein in the liver. Six identified transgenic males were mated with wild-type C57BL/6J females to create a male transgenic colony for the following carcinogen treatments. Age- and litter-matched male offsprings were divided into six groups of transgenic and six groups of wild-type mice. All individual mice were identified according to the digit code and were arbitrarily assigned to treatment groups. All mice were maintained and cared for in accordance with the guidelines established by the National Institute of Health.

Nested-primer PCR

In a previous study, the nested-primer PCR of HBV x gene fragment from mouse tail genomic DNA was demonstrated to be compatible with RT-PCR of mRNA from liver samples in identifying transgenic animals[23]. In this study, only nested-primer PCR was used to screen the transgenic mice from the colony. The PCR primers were designed on human HBV subtype *adr* x gene sequence. The pair of outer primers included the forward,

GTACCGACGATCCCACACGA, and the reverse, ATTAGGCAGACGTGAAAAAG, complementary to position 1375 to 1394 and 1841 to 1822 of the x gene sequence, respectively. The inner primer set consisted of the forward, GAAACAAATGCAGGGCAGCCG CGACTTAG, and the reverse, CAGTCTTTGAAG TATGCCTCAAGGTCGGT, which were complementary to the nucleotide positions 1421 to 1449 and 1719 to 1691 of the sequence, respectively. They generated a DNA product of 439 bp.

The reaction mixture of the first PCR consisted of 2 μL of DNA extract in a total volume of 20 μL, with final concentrations of 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ (10 × PCR buffer, Promega, Madison, WI), a 10 pmol/L concentration of each outer primer, 0.8 U of AmpliTaq DNA polymerase (Sangon, Shanghai, China), and a 10 mmol/L concentration of each dNTP (Promega, Madison, WI). The reaction mixture of the second PCR was identical with the exception of 3 μL of the first reaction products, 10 pmol/L of inner primers and 2.5 U of AmpliTaq DNA polymerase. Reactions with the outer primer set were thermal cycled once at 95℃ for 5 min, 30 times at 94℃for 50 s, 55℃ for 1 min, and then once at 72℃ for 1 min. For the nested PCR product, reactions were thermal cycled once at 94℃ for 5 min, 30 times at 94℃ for 50 s, 60℃ for 50 s, and 72℃ for 1 min, and then once at 72℃ for 10 min. The PCR products were analyzed by electrophoresis on 20 g/L agarose gels, stained with ethidium bromide, and visualized on an UV transilluminator.

Administration of toxins

Both one-week-old male transgenic mice and their wild-type littermates were used for the studies. All dosing was administered by ip injection based on body weight (bw) at the time of treatment. Animals treated with solvent vehicle (DMSO) were used as the controls. In the tumorigenic study with AFB_1, animals were treated with a single dose of AFB_1 at 6 mg/kg bw and were then euthanized either at 24 wk or 52 wk post-treatment. In the tumorigenic studies with cyanotoxins, either nodularin or MC-LR was ip administered to animals at 10 μg/kg bw once a week for 15 wk, and all animals were sacrificed at 24 wk post-treatment. In the co-tumorigenic studies with AFB_1 and cyanotoxins, the animals were initially given a single dose of AFB_1 at 6 mg/kg bw and then, a week later, were treated with 10 μg/kg bw of MC-LR/nodularin once a week for 15 wk. This experiment was terminated at 24 wk after the treatment with AFB_1.

Histopathology

Animals were sacrificed under ethyl ether anesthesia at 24 wk or 52 wk after the initial treatment. The skin, musculature, and internal organs were examined for visible abnormalities, and tissues with tumors and lesions were preserved in 100 mL/L neutral-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin for histopathological alterations and with periodic acid Schiff's reagent for glycogen accumulation in the hepatocyte. All sections were evaluated by two senior pathologists who were blind to the dosing regimen, and focal hepatic lesions were histologically classified into three morphologic entities: hepatocellular foci, hepatocelluar adenomas or hepatocellular carcinomas.

Statistical analysis

Data were expressed as the incidence of hepatic tumors including carcinoma and adenoma. For animals with both liver adenoma and carcinoma, the statistical analysis of liver tumors was based on the worst level of pathology, carcinoma. The incidences of liver tumors induced by different treatments were statistically evaluated by χ^2 test of a 2 × 2 contingency table or Fisher exact test. One-sided P values less than 0.05 were considered statistically significant.

RESULTS

Synergistic hepatotumorigenesis of AFB_1 and HBVx

During the observation period (24 wk or 52 wk), there was no difference with respect to general fitness between DMSO-treated transgenic and wild-type mice. Macroscopic examination at autopsy revealed no visible tumors in these two control groups of animals. In some AFB_1-treated animals, however, liver tumors were usually multifocal and formed large masses (Figure 1-A1). Upon microscopic inspection, neither neoplastic nor dysplastic alterations were recognized in the livers of DMSO-treated transgenic mice at both 24 and 52 wk post-treatment, but a few inflammatory cells were observed at 52 wk. Some animals treated with AFB_1 developed foci of altered hepatocytes, concentrated mainly in centrilobular areas in which foci consisted of relatively large hepatocytes with vacuolations poorly stained with hematoxylin-eosin staining in the cytoplasm (Figure 1-A2). These vacuolations contained glycogen, as shown in periodic acid Schiff's staining (Figure 1-A3). Clusters of small basophilic cells were observed in some AFB_1-treated animals as well. These phenotypic changes were found in both AFB_1-treated transgenic and wild-type animals. Histologically, tumors displayed extensive pleomorphism and a significantly higher mitotic rate than the adjacent normal liver tissue (Figure 1-A4). They generally were composed of enlarged cells with lipid or glycogen in the cytoplasm.

During the 52-wk observation period, 6 of 29 (20.7%) AFB_1-treated transgenic animals developed HCC, whereas only 1 of 15 (6.7%) AFB_1-treated wild-type mice was found to have HCC. In addition, small neoplastic nodules (adenoma) were identified in 7 of 29 (24.1%) AFB_1-treated transgenic animals and 1 of 15 (6.7%) AFB_1-treated wild-type animals at the 52-wk interval. Overall, the incidence of liver tumors reached 44.8% (13/29) in AFB_1-treated transgenic animals during the observation period of 52 wk, which was significantly higher than that in AFB_1-treated wild-type littermates (13.3%) at the same interval ($\chi^2 = 4.36$, $P < 0.05$). In the short-term tumorigenic study (Table 1), no HCC was recognized in any of AFB_1-treated animals at 24 wk post-treatment; basophilic adenoma was identified in 1 of 11 (9.1%) AFB_1-treated transgenic

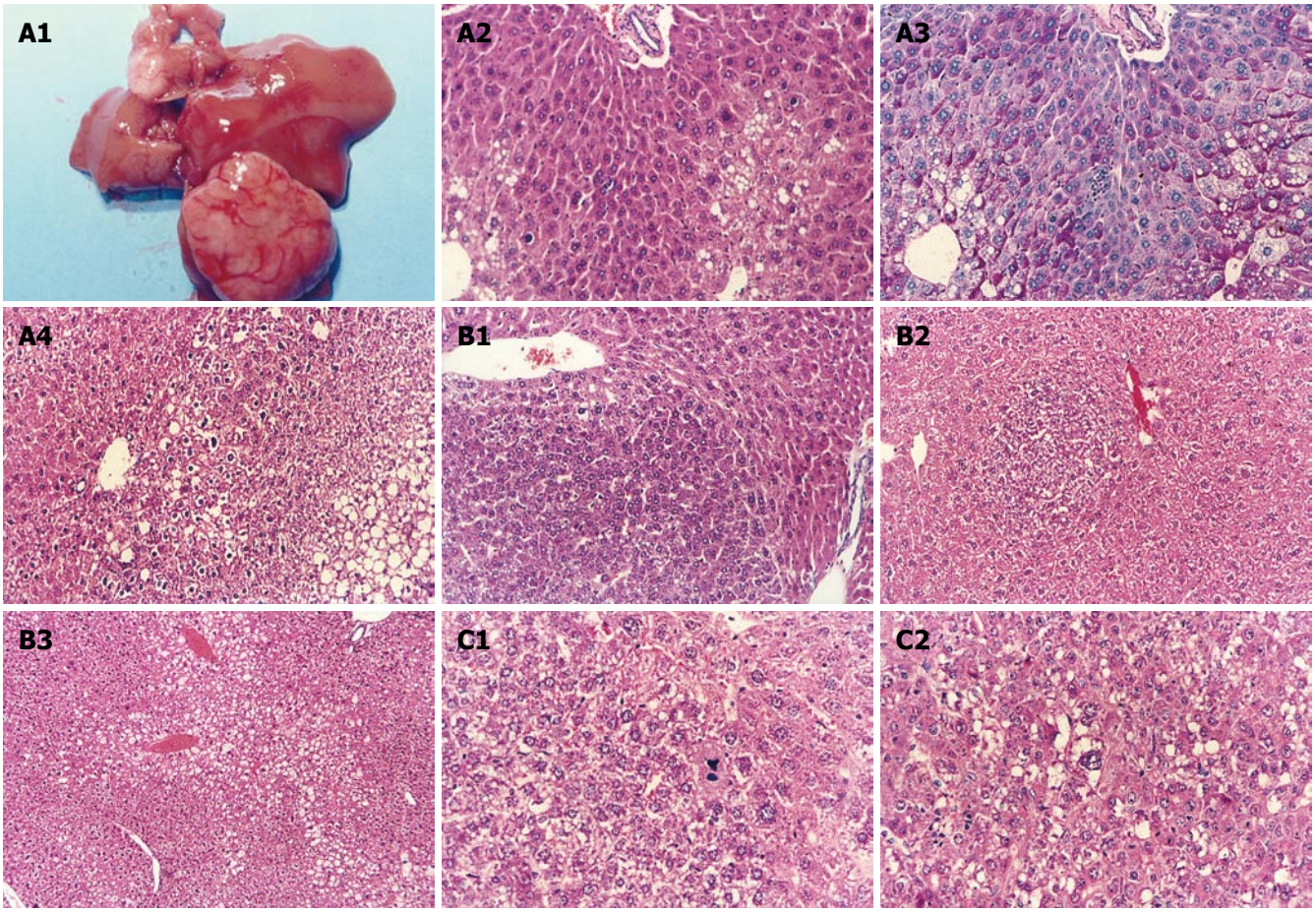


Figure 1 Histopathological alterations identified in liver tissues of mice treated with either AFB_1 (**A**) and cyanobacterial toxins alone (**B**) or in combination (**C**). **A1**: Grossly identified hepatocelluar carcinoma (HCC) in an HBVx gene transgenic mouse at 52 wk post-treatment; **A2**: altered hepatocyte foci in an HBVx gene transgenic mouse (HE × 100); **A3**: periodic acid Schiff's stain for glycogen (× 100) in the continuous liver tissue section of **A2**; **A4**: hepatocellular carcinoma in a wild-type mouse at 52 wk post-treatment (HE × 40). The border appears to be disrupted by neoplastic cells penetrating into the adjacent parenchyma; **B1**: basophilic adenoma in a microcystin-LR-treated wild-type mouse (HE × 40); **B2**: basophilic hepatocyte foci in microcystin-LR-treated transgenic mice (HE × 10); **B3**: clear hepatocyte foci in nodularin-treated wild-type mice (HE × 10); **C1**: HCC in a wild-type mouse treated with both AFB_1 and microcystin-LR (HE × 100). Tumor cells had a great variability in cell and nuclear size, and large cells with large hyperchromatic nuclei were present; **C2**: HCC in a wild-type mouse treated with both AFB_1 and nodularin (HE × 100).

mice and 1 of 20 (5.0%) AFB_1-treated wild-type mice. No statistically significant difference in the incidence of liver tumors was found between these two groups at 24 wk post-treatment. Compared with transgenic animals treated with AFB_1 and sacrificed at 24 wk post-treatment, AFB_1-treated transgenic mice were more likely to develop liver tumors 52 wk after the treatment ($P < 0.05$). In wild-type animals, the AFB_1 treatment did not significantly elevate the risk of developing liver tumors over time.

Contribution of cyanotoxins to the occurrence of AFB_1-induced liver tumors during the short period

To determine if AFB_1 interacts with cyanotoxins to promote the development of liver tumors, wild-type mice were treated with a single injection of AFB_1, followed by repeated injections of either MC-LR or nodularin a week later. Of 21 wild-type mice treated with AFB_1, followed by MC-LR, liver tumors were observed in 4 (19.0%) animals at 24 wk post-treatment (Table 1). Mitotic figures were observed in some tumors. The hepatocytic nuclei were variable in size and chromatin content (dense nuclear chromatin pattern) (Figure 1-C1). By contrast, the incidence of liver tumors in wild-type mice treated with AFB_1 or MC-LR alone was 5.0% (1/20) and 3.9% (1/26) at the same interval, respectively (Table 1). Although the difference in short-term risk of liver tumors was not statistically significant, co-exposure to AFB_1 and MC-LR tended to elevate the risk during the 24-wk period. Alternatively, the incidence of liver tumors in wild-type animals treated with both AFB_1 and nodularin was 33.3% (3/9), which was significantly higher than those in wild-type mice treated with nodularin alone (0%, $P < 0.05$) and slightly higher than those in wild-type mice treated with AFB_1 alone (5.0%, $P = 0.07$) (Table 1). The tumor cells were enlarged and pleomorphic in shape. The nuclei were hyperchromic and exhibited irregular nuclear contour with prominent nucleoli and increased mitotic figures. Small vacuoles found in the dysplastic cells were also observed in the cytoplasm of the neoplastic hepatocytes (Figure 1-C2). This finding suggests a synergistic interaction of AFB_1 and nodularin in the pathogenesis of liver tumors.

In addition to liver tumors, other pathological changes included small eosinophilic, basophilic and clear hepatocellular foci in these groups of animals.

Contribution of cyanotoxins and HBV x gene to the development of liver tumors during the short period

Although a wild-type mouse treated with MC-LR was

Table 1 Incidences of liver tumors caused by exposure to aflatoxin B_1 (AFB_1) and cyanobacterial toxins (CT) alone or in combination in HBV x (HBVx) gene transgenic mice and their wild-type littermates at 24 wk post-treatment

Group	Treatments			*n*	Adenoma (%)	Carcinoma (%)	Total (%)
	HBVx gene	AFB_1	CT				
1	-	-	-	40	0 (0)	0 (0)	0 (0)
2	+	-	-	10	0 (0)	0 (0)	0 (0)
3	-	+	-	20	1 (5.0)	0 (0)	1 (5.0)
4	+	+	-	11	1 (9.1)	0 (0)	1 (9.1)
	HBVx gene	AFB_1	MC-LR				
5	-	-	+	26	1 (3.9)	0 (0)	1 (3.9)
6	+	-	+	11	0 (0)	0 (0)	0 (0)
7	-	+	+	21	2 (9.5)	2 (9.5)	4 (19.0)[a]
8	+	+	+	15	2 (13.3)	1 (6.7)	3 (20.0)[a]
	HBVx gene	AFB_1	NOD				
9	-	-	+	26	0 (0)	0 (0)	0 (0)
10	+	-	+	11	0 (0)	0 (0)	0 (0)
11	-	+	+	9	2 (22.2)	1 (11.1)	3 (33.3)[b,c]
12	+	+	+	11	4 (36.4)	0 (0)	4 (36.4)[d,e,f]

MC-LR: Microcystin-LR; NOD: nodularin. [a]$P < 0.05$ *vs* group 1, [b]$P < 0.01$ *vs* group 1, [c]$P < 0.05$ *vs* group 9, [d]$P < 0.01$ *vs* group 1, [e]$P < 0.05$ *vs* group 3, [f]$P < 0.01$ *vs* group 9.

found to have a basophilic adenoma (Figure 1-B1), no hepatic tumors were observed in all transgenic and other wild-type animals treated with either MC-LR or nodularin. None of DMSO-treated transgenic animals was found to have pathological alterations relevant to tumorigenesis during the 24-wk period. A few small clusters of basophilic and clear hepatocytes were identified in cyanotoxin-treated animals (Figure 1-B2, B3). This finding reveals that cyanotoxins and HBV x gene may play a critical role in the pathogenesis of liver tumors as a tumor promoter rather than an initiator.

Contribution of co-exposure to AFB_1 and cyanotoxins to the pathogenesis of liver tumors in HBV x gene transgenic mice during the short period

As shown in Table 1, transgenic mice treated with AFB_1, followed by MC-LR have a similar risk of liver tumors to their wild-type littermates with the same treatment at 24 wk (20.0% *versus* 19.0%). Consistently, the HBV x gene did not significantly enhance the risk of liver tumors induced by both AFB_1 and nodularin compared to wild-type mice with the same dosing regimen (36.4% *versus* 33.3%). These findings suggest no appreciably combinative hepatotumorgenecity of AFB_1, cyanotoxins and HBV x gene during the 24-wk observation.

DISCUSSION

The nature of coexistence of many types of biotoxins in complex environmental samples, such as food and water, has been reported worldwide. Clear evidence has shown that human populations, particularly HBV carriers in high-risk areas of HCC, are exposed to multiple potent biotoxins, such as aflatoxins and cyanotoxins, through their daily diet and drinking water[24]. To test the epidemiological finding of a combinative risk of HCC associated with co-exposures to AFB_1, cyanotoxins and HBV, we performed a series of tumorigenic studies in HBV x gene transgenic mice treated with AFB_1 and/or cyanotoxins. Although a little inflammation was observed at 52 wk, there was no obvious cell death or regeneration process in DMSO-treated transgenic mice. Even no tumors developed in them during the observation up to 52 wk. Expression of HBV x gene in the liver of AFB_1-treated mice was associated with a greater than 2-fold increase in the incidence of liver tumors at 52 wk post-treatment as compared with AFB_1-treated wild-type littermates. This finding indicates that the HBV X protein functions as a cofactor during AFB_1-mediated hepatocarcinogenesis. The cofactor role of HBV x antigen in the development of hepatocellular carcinoma was also found in the studies of mice carrying HBV x gene exposed to hepatocarcinogen DEN[8,9]. Alternatively, studies of co-tumorgenicity of HBV and AFB_1 using other HBV transgenic mouse models have established that the fragment of HBV genome encoding the entire HBV surface antigen confers a 4- to 20-fold increase in the incidence of liver tumors[11,25].

The underlying mechanism by which the HBV X protein predisposes hepatocytes to the carcinogenic effect of chemical agents has not been fully understood. One possible explanation is the HBVx-induced modulation of specific phase I and II metabolizing enzymes that are involved in bioactivation and elimination of AFB_1. Over-expression of HBV surface antigen and x gene was associated with up-regulated expression of specific cytochrome P450 (CYP) isoforms[10] and responsible for bioactivation of AFB_1 to the ultimate carcinogenic form, exo-AFB_1-8,9-oxide[26]. Additionally, hepatocellular HepG2 cells transfected with HBV demonstrated significant decreases in total GST activity and the level of GST α class[27]. This class of GST is well known to catalyze the reaction in which the AFB_1-8,9-epoxide is detoxified in

the form of glutathionyl-AFB_1 conjugate. Expression of HBV x gene alone, however, was insufficient to induce transactivation of CYP and GST genes or to alter the antioxidant system[28]. Therefore, this possible explanation of the synergistic carcinogenic effect of AFB_1 and HBV x gene remains controversial. A second possible mechanism is the ability of the x gene to inhibit DNA repair, leading to accumulation of DNA mutations induced by chemical carcinogens[29]. HBV X protein has been shown to interact with protein or protein complexes that are directly involved in DNA repair. HBV X protein interferes with the nucleotide excision repair pathway through altering activities of helicases[30,31] or interacting with X-associated protein1 to affect the recognition of bulky lesions in DNA[32]. Another possible mechanism is HBVx transactivation of a wide variety of cellular and viral genes as well as the induction of signaling pathways[7,33]. HBV X protein can induce cell proliferation and deregulate cell-cycle checkpoints, which promotes the survival and proliferation of altered hepatocytes after exposure to a mutagenic agent. HBV X protein transactivates the proto-oncogenes like c jun/fos, c myc and cytokine-encoding genes such as tumor necrosis factor α and transforming growth factor β. On the other hand, HBV X protein can inactivate the cellular tumor suppressor gene product, p53[34]. These findings indicate HBV X protein acts as a tumor promoter in hepatocarcinogenesis. For the virus itself, HBV X protein was reported to enhance the virus replication by activating viral gene expression[35]. Although HBV X protein alone was reported to induce liver tumors in HBVx transgenic mice[36-38], our study showed that HBV X protein alone was insufficient to induce pathologic changes relevant to carcinogenesis, which was consistent with the studies by Lee *et al*[39] and Reifenberg *et al*[40]. The discrepancy may be a result of different genetic backgrounds of transgenic models. In studies showing liver tumors in HBVx transgenic mice, a mouse strain with a very high incidence of spontaneous liver tumors was used[36]. Since our transgenic mice were derived from C57BL/6J mice, the expected incidence of spontaneous liver tumors was less than 4%[38]. In addition, the factor responsible for the difference may include the DNA fragment of HBV genome which was subcloned to the vector. The segment of DNA used in our study contained only the entire coding region of the HBV x gene; by contrast, Yu *et al*[38] used the DNA fragment containing the sequences of HBV x gene, the pre C, and the transcriptional enhancer. Another possibility is the integration site of target gene to the host genome and the copy number of transgene. Our result indicated that the expression of x protein alone predisposes hepatocytes to AFB_1-induced carcinogenesis, although x protein itself does not induce liver tumors.

In the short-term study of the contribution of cyanotoxins alone and in combination with two other factors to the risk of liver tumors, a few altered hepatocellular foci were identified in transgenic and wild-type mice exposed to cyanotoxins alone. With the exception of an MC-LR-treated wild-type mouse with basophilic adenoma, however, no hepatic tumor was found in animals treated with cyanotoxins alone. HBV x gene was not shown to promote the hepatotumorigenicity of cyanotoxins. An important finding in this study, however, was derived from wild-type animals receiving both AFB_1 and cyanotoxins (MC-LR or nodularin). For wild-type animals, the treatment with both AFB_1 and MC-LR was more likely to induce liver tumors than the treatment with one of them during the short-term observation, although the increase in risk was non-significant. Additionally, wild-type mice with the treatment of both AFB_1 and nodularin have a higher risk of liver tumors 24 wk post-exposure than wild-type animals treated with one of them. The difference in risk reached the statistically significant or marginally significant level. These findings suggest that MC-LR and nodularin possess an ability to modulate the development of liver tumors in the AFB_1-initiated animal model. Also, these data proved the previous finding that cyanotoxins are poor initiators and are strong tumor promoters[18,19,22]. Moreover, nodularin was indicated to be a more potent tumor promoter than MC-LR, which is consistent with two-stage carcinogenesis study of modulations of DEN-initiated GST-P-positive foci by cyanotoxins[18,22]. As opposed to what we expected, HBV x gene was not found to further enhance the tumor incidence caused by both AFB_1 and cyanotoxins. These results indicate that tumor promotion of cyanobacterial toxins is stronger than HBV X protein alone during 24-wk period. The tumor promoting activity of cyanotoxins may be attributable to inhibition of protein phosphotase 1 and 2A and subsequent hyperphosphorylation of proteins[41]. The possibility of combinative hepatocarcinogenesis of AFB_1, cyanotoxins and HBV x gene could not been ruled out based on our finding. In this study, the tumorigenic effect of these factors was observed during the short period. Generally, the *in vivo* study of carcinogenesis was carried out in a period of more than a year. Actually, our preliminary study found that two out of four HBV x gene transgenic animals developed liver tumors 52 wk after the treatment with AFB_1, followed by cyanotoxins. The incidences of AFB_1-induced liver tumors were 5.0% and 9.1% in wild-type and transgenic animals, respectively, at 24 wk after the treatment. The same treatment, however, caused a greater than two-fold increase in the incidence in wild-type mice and approximately five-fold increase in transgenic mice at 52 wk post-treatment. Therefore, observation duration is a critical factor in studies of tumorigenicity of potential carcinogens. If the observation period is prolonged, the difference in tumor risk, which was not found significant in the short-term study, may be discernible.

Another limitation of this study was the route of administration of toxins. Repeated injections of cyanotoxins may result in tumor occurrence, although no tumor was observed in almost all animals treated with cyanotoxins. It would be ideal to conduct future studies where the animals are fed AFB_1-contaminated food and cyanotoxin-contaminated drinking water to best fit a model relevant to human exposure.

In conclusion, our study shows that AFB_1 is a strong tumor initiator, and that HBV x gene and nodularin

significantly promote the development of AFB_1-induced liver tumors. Although the significance of the contribution of MC-LR to AFB_1-initiated hepatotumorigenesis is weak, co-exposure to AFB_1 and MC-LR tended to elevate the risk of liver tumors at 24 wk relative to exposure to one of them. HBV x gene could not further increase the contribution of cyanotoxins to AFB_1-initiated liver tumors during the short period. Accordingly, a long-term study of interactive hepatocarcinogenesis of these three factors is needed to confirm the present finding.

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RAPID COMMUNICATION

Alterations of seminal and hormonal parameters: An extrahepatic manifestation of HCV infection?

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Abstract

AIM: To evaluate the possible influences of HCV infection and relative antiviral treatment on seminal parameters and reproductive hormonal serum levels.

METHODS: Ten male patients with HCV-related chronic hepatitis and 16 healthy male volunteers were studied. In all subjects seminal parameters (nemaspermic concentration, progressive motility, morphology) and hormonal levels were determined. Seminal parameters and inhibin B, follicle-stimulating hormone, luteinizing hormone, total and free testosterone, estradiol, prolactine in patients were measured after six and twelve months of antiviral combined (interferon + ribavirin) treatment.

RESULTS: Patients before treatment showed a significantly lower nemaspermic motility and morphology as well as lower inhibin B and free testosterone levels than controls. Inhibin B levels in cases were improved six and 12 mo after treatment in five responders (161.9 ± 52.8 pg/mL versus 101.7 ± 47.0 pg/mL and 143.4 ± 46.1 pg/mL versus 95.4 ± 55.6 pg/mL, respectively). Hormonal pattern of patients did not significantly change after treatment, with the exception of estradiol levels with an initial reduction and an overall subsequent increment (19.7 ± 6.4 pg/mL versus 13.6 ± 5.0 pg/mL versus 17.3 ± 5.7 pg/mL). However in 1-year responders a significant increment of free testosterone (14.2 ± 2.54 pg/mL versus 17.1 ± 2.58 pg/mL) occurred. An impairment of nemaspermic morphology occurred, while other seminal parameters did not change significantly during antiviral treatment.

CONCLUSION: Patients with HCV infection show worse spermatic parameters than controls, suggesting a possible negative influence of virus on spermatogenesis, with further mild impairment during antiviral treatment. However therapy could improve the spermatic function, as suggested by the increased inhibin B levels and improved hormonal pattern in responders. Further studies are needed to confirm these preliminary intriguing results.

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Key words: HCV hepatitis; Seminal parameters; Antiviral treatment; Extrahepatic manifestations

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INTRODUCTION

Hepatitis C virus (HCV) chronic infection is widespread in the world with about 150-180 millions of carriers[1]. Chronic HCV-related hepatitis is a frequent condition with a significant clinical impact due to the rare spontaneous virus disappearance in carriers. Virus transmission occurs predominantly *via* parenteral routes, but is often unclear. Sexual and maternal transmissions are rare (about 5%)[2]. Combined administration of peghilated α-interferon (PEG-IFN) plus ribavirin is the treatment of choice for HCV infection (from 45% to 80% of virus eradication)[3].

HCV is involved in many extrahepatic conditions and at least one of these manifestations is present in 74% of cases (the term "HCV disease" has been used to underline its systemic aspects)[4,5]. The association between extrahepatic diseases and HCV infection has not been definitely proven. Moreover, no organ-specific antibodies (ANA, SMA, anti-LKM) are present in 20%-40% of cases with uncertain role, which might be the results of lymphocyte B stimulation by HCV or the HCV-induced anti-LKM1 recognition of specific antigens[6, 7].

Mixed cryoglobulinemia is the most frequent extrahepatic HCV manifestation (43%-90% of cases)[8, 9]. Other associated diseases include crioglobulinaemic nephropaty and glomerulonephritis[10,11], thyroid diseases (during or after interferon treatment)[12, 13], autoimmune gastritis and

less frequently Sjögren or Sjögren-like syndromes[14], idiopathic pulmonary fibrosis[15], porphyria cutanea tarda[16], lichen planus[17], type 2 diabetes mellitus[18]. Moreover interferon frequently seems to precipitate latent autoimmune gastritis, particularly in females, which is often associated with antityroid antibodies[19].

Very few studies have been performed to evaluate the presence of HCV-RNA in seminal fluid and spermatozoa, particularly in candidates for assisted reproduction techniques, whose seminal parameters and fertility are very poor.

The presence of the virus in the cervico-vaginal secretions has been already demonstrated, while data about its presence in semen are controversial[2]. Indeed polymerase chain reaction (PCR) inhibitors in seminal fluid, especially *Taq* polimerase inhibitors, could interfere with results obtained by this methodology. Levy *et al*[20] suggested that HCV-RNA could be found in males' semen when *Taq* inhibitors have been suppressed by diluitions, thus explaining the previous contradictory results. The authors demonstrated that 30% of the studied males show semen abnormalities (two oligozoospermias and ten oligoasthenoteratozoospermias), confirming the presence of HCV-RNA in the seminal fluid before antiviral treatment for high serum viral load carriers[21]. On the contrary, Debono and co-workers[22] showed that HCV-RNA could not be detected in semen (seminal fluid and spermatozoa) either by PCR or by branched DNA or *in situ* hybridisation[22]. Moreover, these studies have confirmed the low prevalence of HCV sexual transmission.

The aim of this study was to evaluate the seminal parameters (volume, pH, nemaspermic concentration, motility and morphology) and reproductive hormone serum levels including follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactine (PRL), 17-β-estradiol (17-βE), total and free testosterone, inhibin B, in subjects with chronic HCV-related hepatitis before and after six and twelve months of standard antiviral combined therapy (peg-interferon plus ribavirin), in order to verify the possible influences of the virus and/or antiviral treatment on seminal and hormonal variables.

MATERIALS AND METHODS

Patients

Ten consecutive male patients (age range 33-39 years) affected by HCV-related chronic hepatitis, followed at our department, with viral replication (17 240-5 176 100 copies/mL, branched DNA vers.3.0) and increased aminotransferase levels (1.4-4 X) were enrolled in March-April 2003. They showed normal hepatic function (values of bilirubin, albumin and coagulative parameters within ranges) and no ultrasonographic features of portal hypertension. Viral genotypes were 1 (60%), 2 (20%), and 3 (20%). Causes of viral transmission were unknown in all patients. Two subjects (monitored as blood donors) were infected in the last few months, the mean duration of infection was 11.0 ± 2.7 years. Nobody showed either history of cryptorchidism, varicocele or other conditions at risk for infertility, or other causes of liver disease (HBV, alcohol, autoimmunity, hemochromatosis, *etc*) and previous treatment for hepatitis C.

Sixteen male healthy volunteers aged 18-52 years, without history or features of liver disease, were enrolled in the same period and served as the control group.

Table 1 Characteristics of controls and patients before antiviral treatment (mean ± SD)

Parameters	Cases	Controls
Number	10	16
Age (yr)	36.3 ± 2.4	29.7 ± 7.3
Sperm concentration (n/mL)	85.5 ± 63.5	103.6 ± 54.1
Normal motility (%)	45.7 ± 8.8	60.4 ± 6.9
Normal morphology (%)	28.1 ± 9.6	42.0 ± 6.7
Inhibin B (pg/mL)	101.7 ± 47.0	192.8 ± 78.6
Free testosterone (pg/mL)	15.2 ± 2.2	21.4 ± 3.1
Total testosterone (μg/L)	6.72 ± 2.1	6.7 ± 1.3
Prolactine (μg/L)	8.19 ± 5.1	4.6 ± 1.9
LH (mU/mL)	4.02 ± 2.2	2.5 ± 0.7
FSH (mU/mL)	4.81 ± 1.8	3.6 ± 1.4
17-βE (pg/mL)	19.7 ± 6.4	14.9 ± 2.9

A trained blinded operator investigated chemical-physical characteristics of seminal fluid (volume, pH, sperm concentration, motility and morphology) in patients and controls, according to the WHO criteria and recommendations (1992/1999)[23]. The following hormones were measured in HCV patients and controls: FSH, LH, PRL, estradiol, total and free testosterone, inhibin B. FSH, LH and PRL were determined by immunoradiometric assay (IRMA) (FSH: normal values [nv] 1.2-10 mU/mL; intra- and inter-assay coefficients of variation [CV] 7.5%-8.3%; sensitivity 0.18 mU/mL; LH: nv 1.5-7 mU/mL, CV 5.8%-13.8%, sensitivity 0.20 mU/mL; PRL: nv 2-12 ng/mL, CV 2.7%-8.9%, sensitivity 0.5 ng/mL). 17-βE and total/free testosterone levels were determined by radioimmunoassay (RIA) (17-βE: nv 10-40 pg/mL, CV 10.5%-9.1%, sensitivity 1.4 pg/mL; total testosterone: nv 3-10 ng/mL, CV 8.7%-13.7%, sensitivity 0.05 ng/mL; free testosterone: nv 16-41 pg/mL between 20-50 years of age and 9-31 pg/mL > 50 years of age, CV 10.6%-9.9%, sensitivity 0.15 pg/mL). Serum inhibin B was measured by enzyme linked immunosorbent assay (ELISA) (nv 97-330 pg/mL, CV <7%, sensitivity < 15 pg/mL).

The same seminal parameters and hormone levels were determined in cases at 6 and 12 mo after starting antiviral combined treatment with 1.5 μg/kg peg-interferon α2b a week plus ribavirin 800-1200 mg a day (according to weight). The duration of treatment was twelve months.

Statistical analyses were performed using Student's *t*-test and when appropriate, using matched pair t-test. Age-adjustments were performed by a multiple regression model.

RESULTS

The sperm concentration was not significantly different between controls and cases (before antiviral therapy), while nemaspermic motility and morphology were lower in the latter (after age adjustment: $P = 0.0002$ and $P = 0.0003$ respectively). The two patients with more recent HCV infection presented the worse seminal parameters: progressive motility < 40%, normal morphology < 20%. Inhibin B and free testosterone were significantly lower in patients than in controls (after age-adjustment: $P = 0.004$ and $P < 0.001$ respectively) (Table 1).

After six months of combined antiviral treatment, sperm concentration and motility did not significantly

Table 2 Seminal and hormonal parameters in responders and non responders before, during and after treatment (mean ± SD)

	Month 0	Month 6	Month 12	Month 0	Month 6	Month 12
	Non responders			Responders		
Sperm concentration (*n*/mL)	84.2 ± 56.6	69.0 ± 35.5	66.6 ± 36.1	86.9 ± 76.6	35.2 ± 25.8	76.8 ± 75.3
Normal motility (%)	45.2 ± 7.0	51.4 ± 13.9	46.0 ± 7.4	46.2 ± 10.7	37.0 ± 17.8	43.8 ± 24.3
Normal morphology (%)	34.8 ± 7.6	24.6 ± 11.3	26.0 ± 6.8	21.4 ± 6.0	14.4 ± 9.2	21.6 ± 12.8
Inhibin B (pg/mL)	98.0 ± 49.2	166.4 ± 30.3[b]	95.4 ± 55.6	105.3 ± 50.1[a]	157.4 ± 72.7	143.4 ± 46.1
Free Testosterone (pg/mL)	16.2 ± 1.6	16.4 ± 2.0	16.9 ± 3.4	14.2 ± 2.5	14.6 ± 2.8	17.1 ± 2.6[c]
Total testosterone (μg/L)	7.4 ± 2.0	7.9 ± 0.9[d]	6.6 ± 1.0	6.0 ± 2.0	7.3 ± 2.1	6.9 ± 1.0
PRL (μg/L)	7.0 ± 3.8	7.9 ± 1.8	8.9 ± 3.3	9.4 ± 6.3	7.4 ± 3.9	6.5 ± 2.8
LH (mU/mL)	4.2 ± 2.1	3.6 ± 1.8	3.0 ± 1.2	3.8 ± 2.6	4.5 ± 2.5	3.6 ± 0.7
FSH (mU/mL)	4.8 ± 1.7	4.9 ± 1.0	5.7 ± 1.6	4.8 ± 2.1	5.2 ± 0.8	5.1 ± 0.9
17-βE (pg/mL)	19.4 ± 5.3[e]	14.6 ± 5.1	20.2 ± 6.8	20.0 ± 7.9	12.7 ± 5.2	14.5 ± 2.8

[a]$P = 0.039$ (0 *vs* 12 mo); [b]$P < 0.01$ (6 *vs* 12 mo); [c]$P < 0.05$ (0 *vs* 12 mo); [d]$P < 0.05$ (6 *vs* 12 mo); [e]$P < 0.01$ (0 *vs* 6 mo).

change in HCV patients, while further alterations in morphology appeared ($P = 0.01$). At the same time inhibin B levels significantly increased ($P = 0.004$) (Table 2).

Viraemias at the sixth month were negative in seven patients and there were not any significant differences in seminal and hormonal parameters between the former and the three patients with persistent viraemia. Two responders had virological breakthrough at 12 mo and a total of five patients (50%) showed a full response to combined antiviral therapy.

At 12 mo sperm concentration and motility did not significantly change with respect to basal values and 6 mo parameters, and nemaspermic morphology improved slightly in responders. Inhibin B levels increased after six months of therapy both in responders and in non responders. However after twelve months inhibin B values significantly decreased only in non responders (Table 2). Hormone levels were within normal range in all the patients, even if estradiol values significantly decreased at six months with respect to basal values in non responders, but at twelve months its values were raised in non responders and reduced only in responders (Table 2).

A significant increment in free testosterone levels from baseline to the 1 year evaluation occurred (respectively 14.2 ± 2.54 pg/mL versus 17.1 ± 2.58 pg/mL, $P = 0.04$). Furthermore 1-year hormonal pattern was generally better in responders than the corresponding basal levels, even if the values did not reach the statistical significance probably owing to the small number of subjects.

DISCUSSION

Some reports have linked HCV infection to many extrahepatic manifestations, but not all associations have been definitely demonstrated[4]. These data suggest that HCV chronic infection could alter seminal parameters, particularly reduce the percentage of spermatozoa with normal progressive motility and morphology. Patients showed significantly lower levels of free testosterone and inhibin B, a glycoproteic hormone produced by Sertoli cells which is considered a sensible marker of good spermatogenesis[24]. This reduction could be one of the consequences of the altered gametogenesis found in HCV patients.

The combined antiviral therapy could cause a further alteration in spermatic morphology. On the other hand, its antiviral activity could improve spermatogenesis, as demonstrated by the increased inhibin B levels after six and twelve months of therapy in responders. Moreover, after one year of therapy, the responders showed an overall better hormonal pattern than non-responders. Therefore both HCV chronic infection and related antiviral treatment could act on spermatogenesis.

HCV sexual transmission is quite rare and not all authors have identified viral RNA in sperm[20-22]. Previous studies were performed in the context of assisted reproductive techniques, in order to investigate the risk of infection transmission during performing such techniques. The possible interference of HCV with spermatogenesis has not been previously assessed, thus any comparisons with literature are quite difficult.

It has been shown that HCV can stimulate production of reactive oxygen species (ROS) through expression of core protein with resulting *in vitro* and *in vivo* mitochondrial injury, which might explain its hepatic damage, at least in part[25]. Furthermore, oxidative stress could injure genomic integrity in male germinal cells and cause infertility[26]. Thus, oxidative stress induced by HCV-RNA presence in seminal fluid, could directly or indirectly impair spermatogenesis.

These preliminary data show the negative influence of HCV infection on spermatogenesis and the possible beneficial effect of antiviral therapy. A further follow-up of this cohort is therefore necessary. In the present study, some patients did not eliminate HCV after treated for six months, but interferon could act on HCV sufficiently to eliminate adverse seminal effects. This has been demonstrated for HCV-related membranoproliferative glomerulonephritis[11]. In interferon responders, proteinuria decreased from 6.1 g to 1.3 g, while in non-responders a lower decrement was observed.

This is the first report about hormonal and seminal changes in patients with HCV-related chronic hepatitis

during antiviral treatment. Further studies are necessary to confirm these associations and to determine the exact action on seminal and hormonal pattern in order to establish if semen cryoconservation is useful before the therapy in these patients.

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RAPID COMMUNICATION

Production of reactive oxygen species and expression of inducible nitric oxide synthase in rat isolated Kupffer cells stimulated by *Leptospira interrogans* and *Borrelia burgdorferi*

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Abstract

AIM: To evaluate the production of reactive oxygen species (ROS) and the expression of inducible nitric oxide synthase (iNOS) in rat isolated Kupffer cells (KCs) stimulated by *Leptospira interrogans* and *Borrelia burgdorferi*.

METHODS: Rat Kupffer cells were separated by perfusion of the liver with 0.05% collagenase, and purified by Percoll gradients. Purified Kupffer cells were tested *in vitro* with alive *L.interogans* and *B. burgdorferi* preparations. The production of ROS was determined by chemiluminescence, whereas iNOS protein expression was evaluated by Western blot assay using anti-iNOS antibodies.

RESULTS: *B. burgdorferi* and to a less extent *L. interrogans* induced ROS production with a peak 35 min after infection. The chemiluminescence signal progressively diminished and was undetectable by 180 min of incubation. *Leptospirae* and borreliae induced an increased iNOS expression in Kupffer cells that peaked at 6 hours and was still evident 22 h after infection.

CONCLUSION: Both genera of spirochetes induced ROS and iNOS production in rat Kupffer cells. Since the cause of liver damage both in leptospiral as well as in borrelial infections are still unknown, we suggest that leptospira and borrelia damage of the liver can be initially mediated by oxygen radicals, and is then maintained at least in part by nitric oxide.

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Key words: *Leptospira interrogans*; Borrelia burgdorferi; Inducible nitric oxide synthase; Nitric oxide; Reactive oxygen species; Kupffer cells; Chemiluminescence

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INTRODUCTION

The spirochete *Leptospira interrogans* and *Borrelia burgdorferi* are the etiological agents of leptospirosis and Lyme disease, respectively. All spirochetoses share a bacteriemic phase during the early stage of infection. During this phase, *leptospires* can cause hepatitis in humans, resulting in microscopic alterations in the liver, including enlargement of Kupffer cells (KCs)[1]. In Lyme disease, liver function test abnormalities are common but mild, most often not associated with symptoms[2], rarely with hepatitis. The causes of hepatitis and abnormal liver function tests in leptospiral and borrelial infections are not known. Circulating levels of tumor necrosis factor α (TNF-α) have been detected in patients with leptospirosis and are associated with the severity of the disease[3]. Although borrelial invasion may cause direct hepatocyte damage, the acute "viral-like" illness associated with early *B.burgdorferi* dissemination raises the possibility that systemic or local cytokine release as well as other as yet unknown factors may be responsible for hepatic enzyme elevations[2].

The use of *in vitro* and animal models has shown that spirochetes are phagocytized by KCs, the resident macrophages of the liver[4]. Kupffer cells play an important role in host defense due to their ability to phagocyte bacteria and to detoxify endotoxins[5]. Furthermore, activated KCs play a major role in the initiation and maintenance of liver

damage in the above experimental models via production of inflammatory cytokines[6], and trigger a process called the respiratory burst, which involves an increase in cellular oxygen consumption, the production of reactive oxygen species (ROS)[7-10] and nitric oxide (NO) [11]. ROS is cytotoxic for a variety of microorganisms[12], sometimes, however the target of ROS is not limited to the invading pathogens, but can also be extended to host tissues[13], and in the case of the liver, it has been shown that inactivation of KCs prevents liver injury[14]. In this case ROS may cause oxidative damage by peroxidation of membrane phospholipids and alteration in DNA or mithocondrial functions.

Nitric oxide (NO), a gaseous free radical, is a potent biological mediator in a myriad of physiological and pathological events[11]. Abnormalities of NO production have been hypothesized to mediate macrophage cytotoxicity in host defence reactions and to inhibit smooth muscle contraction, hepatocyte metabolism and protein synthesis[15] and to be of central importance in the pathogenesis of many disease processes[16]. Moreover, increased NO production can function as an adaptive response to acute hepatic inflammation and early sepsis[17, 18]. NO production is mediated by members of the nitric oxide synthase (NOS) family[19]. The iNOS isoform is usually activated from inflammatory cytokines and bacterial products, and is responsible for generating high levels of NO[11].

In this study we analyzed the interaction of live *L. interrogans* and *B. burgdorferi* with isolated rat KCs *in vitro*, as far as the production of ROS and iNOS expression is concerned.

MATERIALS AND METHODS

The following spirochete strains were used: *B. burgdorferi* IRS (ATCC 35211) and *L. interrogans* serovar icterohaemorrhagiae (a gift of M. Fabbi, Istituto Zooprofilattico Sperimentale, Pavia, Italy). Borreliae were cultured in Barbour-Stoenner-Kelly (BSK) II medium at 34 ℃, as previously reported[20], whereas leptospirae were grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 30 ℃ under aerobic conditions to a density of about 10^8 bacteria per mL and counted in a Petroff-Hausser counting chamber.

When indicated, opsonized bacteria were prepared as follows: spirochetes were centrifuged and resuspended in RPMI 1640 at a concentration of 4×10^7 bacteria/mL. Bacterial suspensions were incubated for 30 min with pooled sera obtained from Lyme disease and leptospirosis patients, at a concentration of 10%. After incubation, spirochetes were washed twice in RPMI 1640 to remove nonhaderent antibodies.

Kupffer cells (KC) were harvested and separated following the procedure of Smedsrød and Pertoft[21] with some modifications, as previously reported[4]. Male Sprague-Dawley rats (180 g to 200 g body weight) were used as liver donors. The animals were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally), and the livers were perfused through the portal vein, with 500 mL of calcium- and magnesium-free Hanks' balanced salt solution, which was pre-warmed to 37 ℃ and buffered at pH 7.3 with 10 mol/L HEPES (Sigma Chemicals, St. Louis, MO, USA). The effluent from the liver was collected from the inferior vena cava immediately above the sovrahepatic veins. The liver was then perfused with complete Hanks' balanced salt solution containing 0.05% collagenase (type IV; Sigma), pre-warmed to 42 ℃, and subsequentely excised and placed in a Petri dish. The liver capsule was peeled off and the cells were gently dispersed in calcium- and magnesium-free Hanks' balanced salt solution. The cells were then centrifuged at 50 r/min at 4 ℃ for 2 min in a Beckman J6B centrifuge (Beckman Instruments, Palo Alto, CA, USA). The non-parenchymal cell-enriched supernatant was centrifuged at 800 r/min for 10 min, the pellet was resuspended in 40 mL of phosphate-buffered saline (PBS), and portions of 10 mL were layered on top of a preformed two-step Percoll gradient, and centrifuged at 400 r/min for 15 min at 4 ℃. Purified non-parenchymal cells enriched in KCs extended throughout the lower Percoll cushion. The KC-enriched fraction was diluted in PBS and centrifuged at 800 r/min for 10 min. The resulting pellet was resuspended in culture medium (RPMI 1640 with 10% fetal calf serum) at a concentration of 1.0×10^6 cells/mL. A 0.5 mL portion of cell suspension was added to an 8-well culture plate (LAB-TEK, Nalge-Nunc International, Naperville, Ill). Macrophages were selected by allowing them to adhere for 2 h at 37 ℃, in an atmosphere with 50 mL/L. After non-adherent cells were removed by gentle washing, adherent cells were incubated with RPMI 1640 for 24 h before performing further experiments. As a control, immunofluorescence staining was performed with mouse anti-rat CD11b antibody (Research Diagnostics Inc, Flanders, NJ, USA), more than 95% of adherent cells were positive for CD11b macrophage marker.

Reactive oxygen species produced by KCs was determined by chemiluminescence (CL). The determination of ROS was performed as previously reported[6, 10]. Briefly, 0.25 mL of a suspension of purified KCs was added (2×10^6 cells/mL) to selected wells (see below) of a transparent 96-well microtiter plate (Nunclon, Nunc, Denmark). Macrophages were selected by allowing them to adhere for 2 h at 37 ℃, in an atmosphere with 50 mL/L CO_2. After non-adherent cells were removed by gentle washing, adherent cells were incubated with RPMI 1640 overnight before performing the assays. The culture medium was replaced with 200 μL of a fresh one, and 50 μL of a 3 mmol/L luminol solution (5-amino-2,3-dihydro-1,4-phthalazinedione sodium salt, Sigma) in TRIS buffer (0.1 mol/L, pH 7.4) was added to each well followed by 50 μL of a spirochete suspension in culture medium (final ratio 200-2000 bacteria/cell). All reagents were pre-warmed at 37 ℃ before addition. The final luminol concentration was 0.6 mmol/L. Each experiment was performed in triplicate. In order to normalize the CL signal for variables as the number of KCs and cell viability, Kupffer cells stimulated by a fixed concentration (0.8 μmol/L) of phorbol myristate acetate (Sigma) were used as positive control in each run. For each experiment, the background signal of the CL cocktail was evaluated by measuring blank samples constituted by Kupffer cells in growth medium.

The microtiter plate was placed in a luminometer (Luminoskan Ascent, Labsystems, Wallac Oy, Finland) at 37 ℃, and the CL emissions expressed as relative light units were

acquired at 2 min intervals for 3 h, using an integration time of 10 s. Quantitative evaluation of the signals was performed by evaluating the integrated CL signal over the whole measurement period. Then, the CL response of the system was expressed as the ratio between the integrated CL signal of the sample and that of the control. Additional control experiments, in which no luminol was added, were performed. Use of a microtiter plate format allowed to simultaneously follow the kinetics of the chemiluminescent emission for different samples in a simple way. Due to the possible cross-talk between adjacent wells, no more than 12 samples were measured in the same analytical session. Separate experiments carried out with a conventional chemiluminescent system (horseradish peroxidase, luminol, hydrogen peroxide) confirmed that for the emission intensities found in our experiments the cross-talk between different wells was negligible (less than 0.1%) if the wells were separated by at least two empty ones. Mean values of ROS production by KCs were compared using Student's *t*-test. $P < 0.01$ was considered statistically significant.

The determination of iNOS expression by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting was performed as follows. KCs isolated as reported above, were incubated with 200 spirochetes/cell, re-suspended in RPMI 1640, opsonized and unopsonized up to 22 h. As positive controls LPS (10 μg/mL) and INF-γ (100 UI/mL) were used, whereas as negative control any stimuli were omitted. Three different time points were evaluated: 2, 6 and 22 h. At the end of each time point, KCs were scraped, diluted with sample buffer (20% vol/vol Tris 0.5 mol/L, pH 6.8, 2% w/vol SDS, 20% vol/vol glycerol, 10% vol/vol 2-mercaptoethanol, and 0.02% w/vol bromophenol blue) and sonicated. Protein concentration was determined by the Bradford assay. Separation of KC proteins (5 μg/each lane) was performed with a Laemmli buffer system by using a 12% acrylamide gel, as previously described[22]. The Western blot procedure was performed as previously described[23]. Briefly, after electrophoretic transfer, the blots were incubated overnight at room temperature with polyclonal rabbit anti-iNOS antibodies (Affinity Bioreagents, Golden, CO, USA) diluted 1:100 in PBS containing 0.05% (vol/vol) Tween 20. Antigen-antibody complexes were detected using peroxidase-labeled porcine antibodies to rabbit immunoglobulins (DAKO, Copenhagen, Denmark) diluted 1:100 in PBS-Tween. The apparent molecular weight of each band was determined by plotting the positions of rainbow low molecular weight standards (Amersham Biosciences, Little Chalfont, UK). Blots were scanned and the intensity of iNOS bands was determined capturing the images with a Kodak 120 Zoom Digital Camera (Eastman Kodak Company, Rochester, New York, USA) and image processing was performed with Kodak Digital Science 1D 3.0.2 (Eastman Kodak Company, Rochester, New York, USA).

RESULTS

To study ROS production by KCs following incubation with spirochetes, the liver macrophages were incubated alone or with different numbers of spirochetes, and the production of ROS was determined by chemiluminescence. Spirochetes induced ROS production in KCs in a dose-dependent manner (Figure 1). Opsonization of spirochetes increased ROS production, but the increase was not statistically significant (Figure 2), in addition the ROS production induced by borreliae was about twice the generation of oxygen radicals induced by leptospirae ($P < 0.01$) (Figure 2). The time course study of oxygen radical generation showed that ROS was detectable as early as 20 min after the beginning of infection. Leptospirae and borreliae both induced ROS production with a peak 35 min after the addition of spirochetes. The chemiluminescence signal progressively diminished following incubation time and was undetectable for all spirochete preparations at 180 min of incubation (Figure 3). Further time point determinations (4, 6 and 8 h) did not show any ROS production (data not reported).

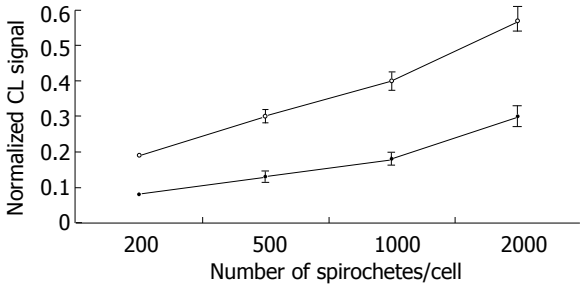


Figure 1 Chemiluminescence (CL) detection of reactive oxygen species generation by Kupffer cells stimulated by L.interrogans and B.burgdorferi. Kupffer cells were incubated in triplicate with borrelia (white circles) and leptospira (black circles) preparations at various bacteria/KC ratio, for 3 h in the presence of 0.6 mM luminol. The chemiluminescence emissions were acquired at 2 min intervals for 3 h. The experiments were repeated three times, and data points represent the mean ± SD of the integrated chemiluminescence signal, normalized to that of the control.

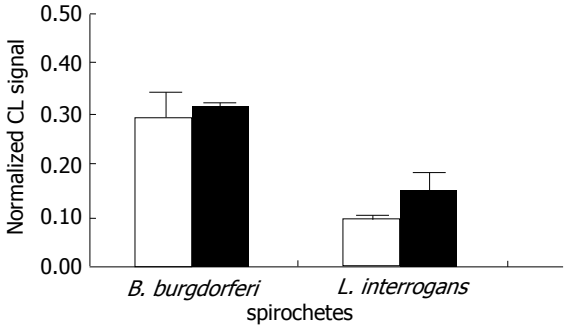


Figure 2 Effect of spirochete opsonization on the chemiluminescence (CL) signal. Each value represents the mean ± SD of three independent experiments carried out at a ratio of 500 spirochetes/KCs using unopsonized (white bars) and opsonized (grey bars) bacteria. Note that the ROS production induced by borreliae was about twice the ROS generation of leptospirae.

To determine whether spirochetes could modify iNOS expression in KCs, iNOS protein was looked for by Western blot assay using polyclonal anti-iNOS antibodies that specifically detect a band of molecular weight of 130 Ku[24]. Kupffer cells were collected 2, 6 and 22 h after bacterial stimulation at the ratio of 200 bacteria/cell. Untreated Kupffer cells as well as LPS- and interferon-γ-stimulated Kupffer cells were also studied. The protein content of each sample was determined and samples of 5

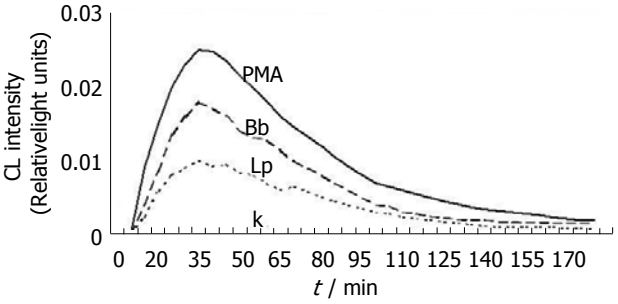


Figure 3 Chemiluminescence kinetic profiles of reactive oxygen radical generation in Kupffer cells treated with L.interrogans and B.burgdorferi. Bacteria were used at a ratio of 2000/KCs. Lp and Bb stand for leptospirae and borreliae, respectively. PMA represents Kupffer cells treated with phorbol myristate acetate, whereas K represents untreated control Kupffer cells.

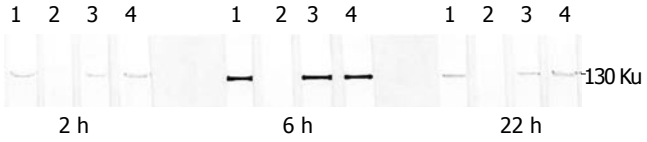


Figure 4 iNOS determination by SDS-polyacrylamide gel electrophoresis and by Western blotting in Kupffer cells stimulated by L. interogans and B. burgdorferi. The 130 ku iNOS protein band was detected by specific polyclonal rabbit anti-iNOS antibodies. The iNOS protein band was evident 2 h after infection. The band peaked 6 h after infection and was still evident 22 h after infection. Lane 1: Kupffer cells stimulated by LPS and interferon-γ (positive control); lane 2: Kupffer cells not stimulated (negative control); lane 3: Kupffer cells stimulated by L.interrogans; lane 4: Kupffer cells stimulated by B.burgdorferi.

μg of protein content were run in each lane and separated by SDS-PAGE. The proteins were then blotted and tested with specific anti-iNOS antibodies. The blots were then scanned and the intensity of iNOS bands was determined capturing the images. The iNOS protein band was evident 2 h after infection. The band peaked 6 h after infection and was still evident 22 h after infection. Six hours after infection, the iNOS band was 5 and 2.5 times more intense than that 2 h and 22 h after infection, respectively. The iNOS protein band was also induced by LPS plus INF-γ (positive controls), whereas the iNOS was not detectable in the absence of stimulating agents (Figure 4). No evident differences in the expression of iNOS protein were seen between KCs stimulated with *B. burgdorferi* and *L. interrogans*, both of which were opsonized and unopsonized.

DISCUSSION

The macrophages of the liver, referred to as Kupffer cells are the most abundant mononuclear phagocytes in the body. The major function of KCs is to clear particulate and foreign materials and micro-organisms from the blood. When activated by antigens or inflammatory stimuli, KCs release superoxide anions, hydrogen peroxide, nitric oxide and hydrolytic enzymes. Kupffer cells also release a number of different immunoregulatory and inflammatory cytokines, including interleukin (IL-1), IL-6, tumor necrosis factor α, interferon-γ[25, 26].

Leptospirae and borreliae are phagocytosed by the Kupffer cells in the absence of opsonizing antibodies (Marangoni *et al* personal Communication VIII International Conference on Lyme borreliosis, Munich, 20-22 June, 1999). The pro-inflammatory cytokine TNF-α is produced by Kupffer cells treated with spirochetes and leptospirae have about twice the stimulatory effect of borreliae[6]. Leptospiral lipo-oligo-saccharide plays a pivotal role in the TNF-α release[6].

The generation of ROS by macrophages and the expression of iNOS occur during the phagocytosis of bacteria, fungi and protozoa[27-29]. In the present work, we comparatively studied the ability of KCs to generate ROS and to express iNOS when stimulated by *L. interrogans* and *B. burgdorferi*. Our findings showed that both the genera of spirochetes induced ROS production in KCs. The use of such a very sensitive method as CL allowed us to demonstrate that when the same amount of bacteria was employed ROS production induced by borreliae was about twice the generation of oxygen radicals induced by leptospirae. This could be partly due to the different size of the bacteria, *B. burgdorferi* being longer and wider than *L. interrogans*. The production of ROS had a maximum 35 min after infection and was undetectable 180 min after infection. The transient feature of the oxidative burst is well documented in monocytes and macrophages, in particular, a similar short-lived and self-limiting oxidative response has also previously been observed during phagocytosis of *Salmonella* by mouse peritoneal macrophages[30, 31] and during the phagocytosis of *T. pallidum* by Kupffer cells[9]. In this study, opsonized leptospirae as well as opsonized borreliae did not enhance ROS production by Kupffer cells in comparison with non-opsonized bacteria. In general, opsonization enhances phagocytosis of bacteria and consequently the production of ROS. However, it has been previously demonstrated (Marangoni *et al* personal Communication VIII International Conference on Lyme borreliosis, Munich, 20-22 June, 1999) that opsonization of leptospirae and borreliae does not enhance phagocytosis by Kupffer cells. Kupffer cells phagocytose leptospirae and borreliae at the same rate in the presence and in the absence of opsonizing antibodies. This probably comes from a constitutional attitude of these bacteria that are not able to escape phagocytosis, differently from *T. pallidum*, a spirochete able to evade phagocytosis by macrophages to a certain extent.

Regarding iNOS, we established that both borreliae and leptospirae induced iNOS expression by Kupffer cells and observed no substantial differences between the two genera of spirochetes in inducing iNOS production.

iNOS is regulated at the transcriptional level of the gene and once induced, iNOS production is maintained for several hours or days[24]. Kupffer cells produce nitrite and nitrate, the end stable products of NO pathway and the overproduction of NO by hepatic cells play a major role in hepatic injury and necrosis[32] associated with endotoxic shock. Kupffer cells also synthetize several cytokines in response to LPS which in turn stimulate the hepatocytes to produce NO[33].

The present *in vitro* investigation demonstrated that

both borreliae and leptospirae could induce iNOS in rat Kupffer cells and that iNOS production was delayed with respect to ROS production. The *in vivo* exposure of KCs to these bacteria, as in the case of leptospirosis and Lyme disease, could lead to a maintained production of NO. In fact, iNOS produces NO from hours to days and overproduction of NO can stimulate neighbouring hepatocytes to generate NO[28] in the liver, resulting in the production of NO over a long period of time after exposure of KCs to leptospirae and borreliae.

In conclusion, our data suggest that a factor responsible for hepatic enzyme elevations in leptospirosis as well as in Lyme disease might be a sequential mechanism of damage initiated by oxygen radicals produced in KCs and maintained by NO production in KCs and likely in hepatocytes.

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RAPID COMMUNICATION

Depressed-type (0-IIc) colorectal neoplasm in patients with family history of first-degree relatives with colorectal cancer: A cross-sectional study

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Abstract

AIM: To investigate the correlation of depressed-type (0-IIc) colorectal neoplasm and family history of first-degree relatives (FDR) with colorectal cancer (CRC).

METHODS: This cross-sectional study was conducted from June 2000 to October 2002 at National Cancer Center Hospital East. Eligible patients undergoing initial total colonoscopy were surveyed regarding family history of CRC among FDR by a questionnaire prior to colonoscopic examinations. All endoscopic findings during colonoscopy were recorded and the macroscopic classification of the early stage neoplasm/cancer was classified into two types (0-IIc *vs* non 0-IIc). Odds ratios (OR) and 95% confidence intervals (CI) were calculated by univariate and multivariate logistic regression to estimate the association between macroscopic features and clinicopathological data including gender, age, and family history of FDR with CRC.

RESULTS: The OR of an association between family history of FDR with CRC and overall early stage neoplasm adjusted by gender and age was 1.85 (95% CI: 1.31-2.61, P = 0.0004), that for non 0-IIc neoplasm was 1.71 (95% CI: 1.22-2.41, P = 0.0017) and for 0-IIc colorectal neoplasm was 2.78 (95% CI: 1.49-5.16, P = 0.0031).

CONCLUSION: Our study shows a significant association between a family history of FDR with CRC and 0-IIc colorectal neoplasm. When patients with a family history of FDR with CRC undergo colonoscopy, colonoscopists should check carefully for not only polypoid, but also depressed-type (0-IIc) lesions.

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Key words: Depressed-type; Family history; Colorectal cancer; First-degree relative; Colonoscopy

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INTRODUCTION

In Japan, colorectal cancer (CRC) is the third most important cause of cancer mortality and the incidence of CRC is increasing gradually[1]. The prognosis for patients with CRC is strictly dependent on early detection of premalignant and malignant lesions. Familial risk management is one of the important strategies for CRC prevention. There is evidence from cohort and case-control studies that people with close relatives with CRC have an increased risk of CRC and develop the disease at a younger age than those without a family history of CRC[2-4]. In clinical guidelines and rationale for CRC screening and surveillance in USA, approximate lifetime risk of CRC, when a first-degree relative (FDR) was affected with large bowel malignancy, was found to increase by 2-3 fold[5]. In addition, there is a report that a family history of CRC is a strong risk factor for adenoma growth[6,7].

However, previous studies concerning familial risk for CRC did not investigate the data from the perspective of macroscopic classifications of colorectal neoplasm. As the Paris endoscopic classification of superficial neoplastic lesions in the colon proposed, special attention is attached to depressed-type (0-IIc) lesions. Even when the diameter is small, 0-IIc lesions are often at a more advanced stage of neoplasia, with deeper invasion than other types of lesion[8,9]. However, to our knowledge, whether the risk of 0-IIc colorectal neoplasm is correlated with family history of FDR with CRC has not been investigated previously.

In this study, we therefore investigated the correlation of 0-IIc colorectal neoplasm and family history of FDR with CRC.

MATERIALS AND METHODS

Eligibility and exclusion criteria

Between June 2000 and October 2002, all patients undergoing initial total colonoscopy at National Cancer Center Hospital East were screened for eligibility for this study. The exclusion criteria were age < 50 years old, past history of surgical resection for CRC, past history of endoscopic treatment for colorectal neoplasm, family history of familial adenomatous polyposis or hereditary non-polyposis colorectal cancer, past history of inflammatory bowel disease and presence of either submucosal tumor, metastatic colorectal tumor or ischemic colitis. Informed consent was obtained from all patients.

Questionnaire of family history and past history

All patients had been asked for family history of CRC among FDR of patients by a questionnaire prior to colonoscopic examinations. Colonoscopists were kept unaware of this information until the end of the examinations.

Total colonoscopy

A preparatory solution of electrolytes and polyethylene glycol was administered orally to each patient. An anti-cholinergic agent was administered intramuscularly before each examination to prevent persistent colonic spasms, if there was no contraindication to its use. Total colonoscopies were performed using magnifying colonoscopes (CF200Z, CF240Z; Olympus Optical Co. Ltd, Tokyo, Japan). All detected lesions were diagnosed by magnifying colonoscopy using a non-biopsy technique to differentiate between hyperplastic polyps and adenomas[10,11]. When any neoplasm was suspected, 0.2% indigo carmine dye was sprayed on the area to highlight the lesions. All detected lesions were finally diagnosed by magnification with chromoendoscopy, and the lesions diagnosed as neoplasm were removed or biopsied to evaluate the histological findings. Lesions, diagnosed as hyperplastic polyps macroscopically or histologically, were excluded from this analysis.

Histological and macroscopic evaluation

Histological diagnoses were based on the classification of the World Health Organization (WHO)[12]. Intramucosal neoplasms, including intramucosal carcinoma and submucosal cancer that had spread through the muscularis mucosa into the submucosa, were defined as early stage neoplasm/cancer. Malignant lesions involving the muscularis propria or beyond on histological examination were classified as advanced cancer.

All endoscopic findings during colonoscopy were recorded at each examination. The macroscopic classification of early stage neoplasm/cancer was classified into two types (0-IIc *vs* non 0-IIc) using the system proposed by the Japanese Society for Cancer of the Colon and Rectum[13], which is nearly similar to the Paris endoscopic classification proposed in 2003[8]. In this study, type 0-IIc lesions included the combined type (0-IIc+IIa, 0-IIa+IIc, 0-Is+IIc)[9] and laterally spreading flat-type tumor[14,15]; so called "LST non-granular type" as described by Kudo in 1993[16]. Other macroscopic type lesions were classified as the non 0-IIc type.

Table 1 Initial colonoscopic findings of the patients

Patients without neoplasm	579 (36.5%)
Patients with neoplasm	1007 (63.5%)
Patients with only early stage neoplasm/cancer	651 (41.0%)
Patients with only advanced cancer	206 (13.0%)
Patients with both early stage neoplasm/cancer and advanced cancer	150 (9.5%)

Statistical analysis

Odds ratios (OR) and 95% confidence intervals (CI) were calculated by univariate and multivariate logistic regression to estimate the association between macroscopic features (early stage neoplasm/cancer, 0-IIc or non 0-IIc) and clinicopathological data including gender, age, family history of FDR with CRC. Two-sided *P* values less than 0.05 were considered statistically significant. Analyses were performed with Stata software (Version 9.0 for Windows, StataCorp LP. TX, USA).

RESULTS

A total of 2079 patients who underwent initial colonoscopy were screened; 1586 were enrolled in this study. Of 493 excluded patients, 297 were less than 50 years old, 84 had a past history of surgical resection for CRC, 62 had a past history of endoscopic treatment for colorectal neoplasm, 7 had a family history of familial adenomatous polyposis or hereditary non-polyposis colorectal cancer, 4 had inflammatory bowel disease; and the rest were excluded because of the presence of either submucosal tumor, metastatic colorectal tumor or ischemic colitis.

Patient characteristics

The study consisted of 1586 patients, including 1002 (63.2%) men and 584 (36.8%) women, with median age 63 (range, 50-96) years. There were 159 (10.0%) patients with a family history of FDR with CRC.

Detected neoplasm during colonoscopy

In 579 patients (36.5%), there were no neoplastic lesions detected on the initial colonoscopy. Colorectal neoplasm was found in 1 007 (63.5%) patients, among whom 651 (41.0%) patients had only early stage neoplasm/cancer, 206 (13.0%) patients had only advanced cancer, and 150 (9.5%) patients had both (Table 1). Furthermore, 775 (48.9%) patients had at least one non 0-IIc lesion and 63 (4.0%) patients had at least one 0-IIc lesion. The prevalence of 0-IIc colorectal neoplasm was 4.6% (95% CI: 3.3-5.9) in men and 2.9% (95% CI: 1.5-4.3) in women.

Table 2 Odds ratio (OR) for early stage colorectal neoplasm calculated by univariate and multivariate analyses

	No. of early stage neoplasm	Univariate analysis			Multivariate analysis		
	(*n* = 801)	Crude OR	95%CI	*P*-value	Adjusted OR	95%CI	*P*-value
Gender							
Female	230	1	1.66-2.51	<0.0001	1	1.64-2.50	< 0.0001
Male	571	2.04			2.03		
Age (yr)							
< 60	248	1	1.04-1.58	0.018	1	0.96-1.47	0.11
⩾ 60	553	1.29			1.19		
Familyhistory of FDR with CRC							
No	701	1	1.25-2.46	0.001	1	1.31-2.61	0.000
Yes	100	1.76			1.85		

Table 3 Odds ratio (OR) for non 0-IIc colorectal neoplasm calculated by univariate and multivariate analyses

	No. of non 0-IIc lesions	Univariate analysis			Multivariate analysis		
	(*n* = 775)	Crude OR	95%CI	*P*-value	Adjusted OR	95%CI	*P*-value
Gender							
Female	221	1	1.65-2.50	< 0.0001	1	1.63-2.49	< 0.0001
Male	554	2.03			2.02		
Age (yr)							
< 60	240	1	1.03-1.57	0.023	1	0.95-1.45	0.14
⩾ 60	535	1.27			1.18		
Family history of FDR with CRC							
No	680	1	1.17-2.28	0.004	1	1.22-2.41	0.002
Yes	95	1.63			1.71		

Table 4 Odds ratio (OR) for 0-IIc colorectal neoplasm calculated by univariate and multivariate analyses

	No. of 0-IIc lesions	Univariate analysis			Multivariate analysis		
	(*n* = 63)	Crude OR	95%CI	*P*-value	Adjusted OR	95%CI	*P*-value
Gender							
Female	17	1	0.91-2.83	0.09	1	0.92-2.90	0.08
Male	46	1.61			1.63		
Age (yr)							
< 60	20	1	0.64-1.89	0.73	1	0.61-1.82	0.86
⩾ 60	43	1.1			1.05		
Family history of FDR with CRC							
No	49	1	1.46-5.04	0.004	1	1.49-5.16	0.003
Yes	14	2.72			2.78		

In terms of age, the prevalence was 4.1% (95%CI: 2.9-5.3) in men and 3.7% (95% CI: 2.1-5.3) in women. These data showed that there was no statistical difference in frequency of 0-IIc colorectal neoplasm among gender and age. In addition, there were 14 (8.8%) patients with 0-IIc lesions and a family history of FDR with CRC.

Association between early stage, non 0-IIc, 0-IIc colorectal neoplasm and variables

Univariate analysis demonstrated that gender, age, and the family history of FDR with CRC were significantly associated with the prevalence of early stage and non 0-IIc colorectal neoplasm. On multivariate analysis, gender and the family history of FDR with CRC were significantly correlated with early stage and non 0-IIc colorectal neoplasm, although there was no significant association between non 0-IIc colorectal neoplasm and age (Tables 2 and 3). Univariate and multivariate analyses demonstrated that only a family history of FDR with CRC was significantly correlated with 0-IIc colorectal neoplasm, whereas gender and age were not found to be significant (Table 4). The OR of association between 0-IIc colorectal neoplasm and family history of FDR with CRC adjusted by gender and age was higher than that of non 0-IIc

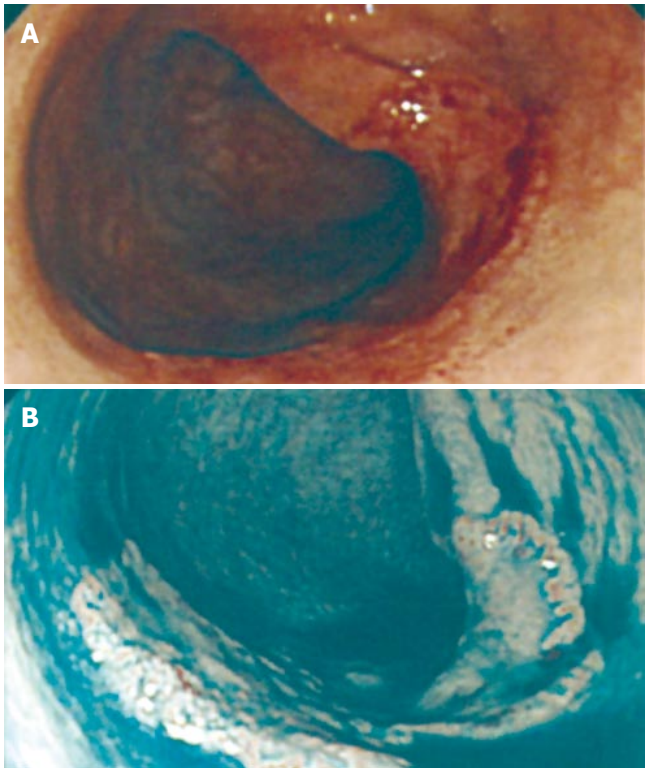


Figure 1 Superficial depressed lesion (0-IIc). **A**: A superficial depressed lesion (0-IIc) of the transverse colon, 27 mm in diameter. Initially it was recognized as a broad patch of erythema and deformity of the haustrum. The patient demonstrated a family history of CRC. His mother had a past history of sigmoid colon cancer at age of 65 years. **B**: After dye spraying, an absolutely depressed area could be clearly identified, with a slightly elevated margin.

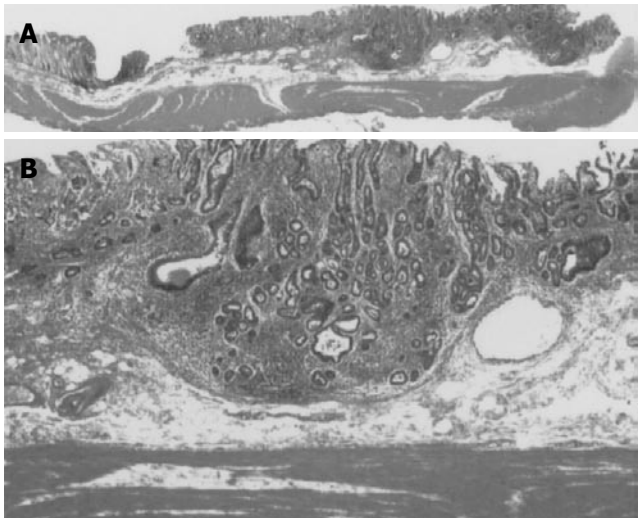


Figure 2 Histological findings. **A**: Low-power histological view of a cut section of the resected specimen confirmed Dukes' A (T1 stage) carcinoma with invasion to the submucosa. **B**: High-power histological view confirmed a well differentiated adenocarcinoma invading 1000 μm below the muscularis mucosa.

colorectal neoplasm (OR = 2.78, 95% CI: 1.49-5.16, P = 0.0031 *vs* OR = 1.71, 95% CI: 1.22-2.41, P = 0.0017).

DISCUSSION

Colorectal neoplasm with superficial morphology is broadly classified into two types, such as polypoid type and non-polypoid type[8,13]. In terms of polypoid type neoplasm, the adenoma-carcinoma sequence has formed the rationale for CRC screening and prevention in Western countries[5,17]. In addition, it is also known that familial risk management is one of the important strategies for CRC prevention due to its associations with bowel malignancy[5] and adenoma growth[6,7]. Almendingen *et al*[7] reported a significant association between family history of FDR with CRC and adenoma growth (adjusted OR = 3.9, 95% CI: 1.2-13.4). In the present study, family history of FDR with CRC was significantly correlated with non 0-IIc colorectal neoplasm (OR = 1.71, 95% CI: 1.22-2.41, P = 0.0017), which tends to show a result similar to previous reports. However, these data are mainly based on the polypoid type colorectal neoplasm.

As the Paris endoscopic classification of superficial neoplastic lesions in the colon proposed, special attention should be focused on depressed-type 0-IIc lesions[8], which are now widely recognized in Western countries as well as in Japan. This recognition has important implication, as the proportion of CRC is likely to be higher in these lesions than in other types, and these lesions tend to be more advanced at the time of diagnosis, despite being smaller[18-23]. Furthermore, it is certainly more difficult to detect 0-IIc colorectal neoplasm than polypoid type lesions during colonoscopy. They usually appear only as patches of erythema or irregularity of the mucosal fold (Figures 1 and 2), so colonoscopists should develop an understanding of the 0-IIc lesion[9]. At the same time, it is very important to identify risk factors for 0-IIc colorectal neoplasm, so if patients with some risk factors for these lesions undergo colonoscopy, we could be more alert to recognizing the lesions. However, it has not been reported whether the risk of 0-IIc colorectal neoplasm is correlated with any patient characteristics, including family history of FDR with CRC.

In the present study, 0-IIc colorectal neoplasm was detected in 63 patients (4.0%), which is nearly similar to data (1.94%-5%) reported by other institutes in Japan[8,9]. More importantly, our study showed a significant correlation with family history of FDR with CRC and 0-IIc colorectal neoplasm (OR = 2.78, 95% CI: 1.49-5.16, P =0.0031), suggesting the possibility of a genetic contribution to the occurrence of 0-IIc colorectal neoplasm. Recently, two major pathways for colorectal neoplasm are highly suspected. Compared to the polypoid lesions arisen in the pathway of adenoma-carcinoma sequence, depressed-type (0-IIc) lesions are proposed to arise in *de novo* pathway and show rapid growth. Molecular biology also suggests that depressed-type lesions are likely to have early *P53* and delayed K-*ras* mutations distinct from the former pathway[24,25]. Moreover, there tends to be high microsatellite instability occurrence in *de novo* cancers compared to early cancers with adenoma[25]. It is also noteworthy that Ricciardiello *et al*[26] have reported there is higher degree of microsatellite instability among patients with family history of FDR with CRC. These results may support our positive association between depressed-type colorectal neoplasm and family history of FDR with CRC.

In addition, the pathway of depressed-type lesions evolving rapidly into a small flat invasive carcinoma is hypothesized to be a major route in hereditary non-polyposis colon cancer (HNPCC)[8], for which the genes hMSH2, hMLH1, and others have been identified as being responsible. In our study, multivariate analysis stratified by age showed higher OR of association between 0-IIc colorectal neoplasm and family history of FDR with CRC in patients before age of 60 years than those after age of 60 years (OR = 4.06, 95% CI: 1.48-11.14, $P = 0.006$ *vs* OR = 2.28, 95% CI: 1.02-5.06, $P = 0.04$), whereas our colleagues previously reported that depressed-type lesions were predominant in the right colon[27]. These characteristics are similar to HNPCC, and further molecular genetic research for depressed-type 0-IIc lesions and HNPCC is required. However, to our knowledge, the association between a family history of FDR with CRC and 0-IIc colorectal neoplasm has not been investigated previously even on NPS (National Polyp Study in USA). The limitation of this study is that the prevalence of patients with neoplasm in this study was higher (63.5%) than that in the general population because of a characteristic of our cancer center hospital, because even we selected the patients who undergo initial total colonoscopy at our hospital, many patients are introduced from general hospital for further examination because of suspicion of colorectal cancer. Although the statistical analysis in this study could avoid these biases, this association should be confirmed in a prospective multicenter study.

In conclusion, the present study demonstrates a significant association between the family history of FDR with CRC and 0-IIc colorectal neoplasm. When patients with a family history of FDR with CRC undergo colonoscopy, colonoscopists should pay attention to not only polypoid, but also depressed-type (0-IIc) lesions.

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RAPID COMMUNICATION

Hydrogen and methane gases are frequently detected in the stomach

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Abstract

AIM: To investigate the incidence of bacterial overgrowth in the stomach by using a new endoscopic method in which intragastric hydrogen and methane gases are collected and analyzed.

METHODS: Studies were performed in 490 consecutive patients undergoing esophagogastroscopy. At endoscopy, we intubated the stomach without inflation by air, and 20 mL of intragastric gas was collected through the biopsy channel using a 30 mL syringe. Intragastric hydrogen and methane concentrations were immediately measured by gaschromatography. *H pylori* infection was also determined by serology.

RESULTS: Most of intragastric hydrogen and methane levels were less than 15 ppm (parts per million). The median hydrogen and methane values (interquartile range) were 3 (1-8) ppm and 2 (1-5) ppm, respectively. The high hydrogen and methane levels for indication of fermentation were decided if the patient had the values more than 90 percentile range in each sample. When a patient had a high level of hydrogen or methane in one or more samples, the patient was considered to have fermentation. The overall incidence of intragastric fermentation was 15.4% (73/473). Intragastric methane levels were higher in the postoperative group than in other groups. None of the mean hydrogen or methane values was related to *H pylori* infection.

CONCLUSION: Hydrogen and methane gases are more frequently detected in the stomach than expected, regardless of the presence of abdominal symptoms. Previous gastric surgery influences on the growth of methane-producing bacteria in the fasting stomach.

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Key words: Intragastric gases; Hydrogen; Methane; *Helicobacter pylori*

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INTRODUCTION

Hydrogen breath tests have been used to evaluate intestinal transit, bacterial overgrowth, and disaccharidase deficiency[1-8]. As hydrogen production increases when a small amount of carbohydrate is supplied to colonic bacteria, the measurement of breath hydrogen concentration has been proposed as an indicator of carbohydrate malabsorption[2]. Similarly, breath methane excretion, which reflects an indirect measurement of the metabolism of the anaerobic colonic flora, has been measured[9,10]. Methanogenic bacteria utilize hydrogen, carbon dioxide, and then synthesize methane[11]. All methane absorbed from the colon reaches the lung and excretes into the breath[12]. If the fermentation occurs in the stomach, we can detect intragastric hydrogen and/or methane gas. We therefore attempted to collect intragastric gas endoscopically and measure the intragastric hydrogen and methane levels in order to determine the bacterial overgrowth in the stomach.

MATERIALS AND METHODS

Patients

Studies were performed in 490 consecutive patients (160 men and 315 women, 19-85 years old) undergoing upper endoscopy. None of the patients had a history of use of proton pump inhibitor (PPI), H_2-receptor antagonist (H_2-RA), antibiotics, steroids, or nonsteroidal anti-inflammatory drugs for a period of at least six month before the investigation. Twelve patients had a previous Billroth I

partial gastrectomy.

Collection of intragastric gas samples

Endoscopy was performed after a topical anesthesia gargle. At the time of endoscopic examination, we intubated the stomach without inflation by air, and 20 mL of intragastric gas was collected through the biopsy channel using a 30-mL syringe. The first 5 mL was discarded for reduction of dead-space error. Intragastric hydrogen and methane concentrations were immediately measured by gaschromatography using Breath Analyzer TGA-2000 (TERAMECS Co. Ltd. Kyoto) and expressed in parts per million (ppm). Linear accuracy response range was 2 to 150 ppm. After collecting an intragastric gas sample, the endoscopist inflated the stomach by air and observed the gastric mucosa.

Determination of H pylori status

At endoscopy, serum *H pylori* IgG antibody titers were measured with an ELISA method (HM-CAP). A value of >2.2 was considered seropositive and a value of <1.9 was considered seronegative. Patients with a value of neither less than 1.9 nor more than 2.2 were excluded from this study.

Statistical analysis

Data of intragastric hydrogen and methane were presented as medians, with interquartile ranges because they were not normally distributed. Comparisons of groups were made using the Mann-Whitney *U* test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Incidence of high hydrogen and methane value in the stomach

Seventeen patients were dropped from this study because their serum *H pylori* IgG antibody titers were indeterminate. Figures 1 and 2 show the distribution of intragastric hydrogen and methane levels in the remaining 473 subjects, respectively. Most of the levels were less than 15 ppm. Overall, the median hydrogen and methane values (interquartile range) were 3 (1-8) ppm and 2 (1-5) ppm, respectively. The high hydrogen and methane levels for indication of fermentation were decided if the patient had the values more than 90 percentile range in each sample. Based on this definition, high hydrogen levels were defined as ⩾21 ppm and high methane values as ⩾8 ppm. In this study, when a patient had a high level of hydrogen or methane in one or more samples, the patient was considered to have fermentation. The overall incidence of intragastric fermentation was 15.4% (73/473). The incidence of intragastric fermentation determined by the intragastric hydrogen level was 11.0% (52/473), whereas those determined by the intragastric methane level was 10.8% (51/473). Of 73 patients with intragastric fermentation, gastric cancer was found in 2 (2/4, 50%), gastric ulcer in 4 (4/38, 11%), duodenal ulcer in 3 (3/15, 20%), previous gastric surgery in 3 (3/12, 25%), and others in 61 patients (61/404, 15%).

Peptic ulcer and intragastric gas concentrations

Intragastric hydrogen and methane values in relation to endoscopic diagnosis are summarized in Table 1. Intragastric methane levels were significantly higher in the postoperative group than in the gastric ulcer group and in the other groups. Intragastric hydrogen levels were lower in the gastric ulcer group than in other groups but this did not reach statistical significance.

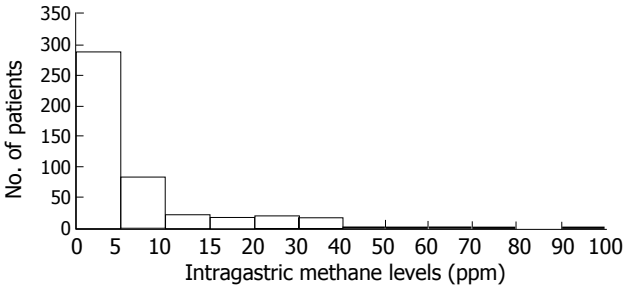


Figure 1 Distribution of intragastric hydrogen levels in all 473 subjects.

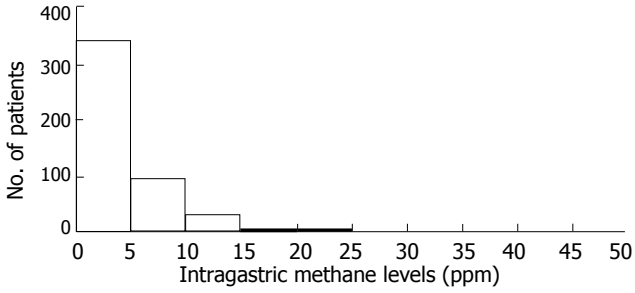


Figure 2 Distribution of intragastric methane levels in all 473 subjects.

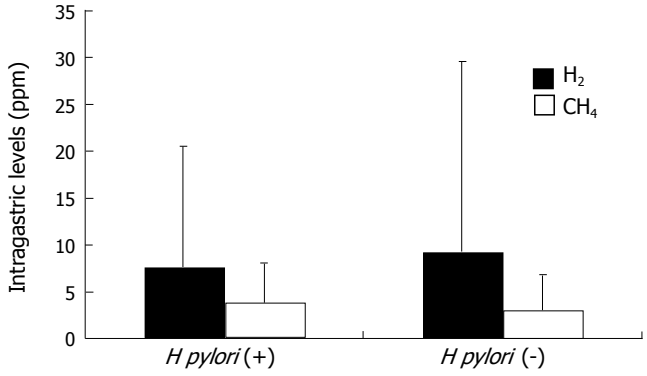


Figure 3 Intragastric hydrogen and methane levels in relation to *H pylori* status.

Table 1 Intragastric hydrogen and methane levels in relation to endoscopic findings

	Gastric ulcer	Duodenal ulcer	Postoperative stomach	Others
No.of patients	38	15	12	404
H2 (ppm)	6.8 ± 10.7*	10.9 ± 17.2	11.0 ± 13.9	8.2 ± 16.9
P values *vs**	*	0.15	0.14	0.31
CH4 (ppm)	3.7 ± 5.6	3.7 ± 3.6	8.1 ± 12.6**	3.4 ± 4.1
P values *vs***	0.047	0.1	**	0.0002

H pylori infection and intragastric gas concentrations

Figure 3 shows the means of intragastric hydrogen and methane concentrations by *H pylori* status. None of the mean values were related to *H pylori* infection.

DISCUSSION

Before the discovery of *H pylori* infection in 1983[13], it was demonstrated by many investigators that an increased number of bacteria had been found in the stomach in patients with achlorhydria or hypochlorhydria[14]. The type and numbers of microbial flora present in the stomach are affected by gastric pH[15-17], and a rise in intragastric pH has often been associated with an increased number of bacteria in gastric juice[18-20]. Atrophic gastritis is the most common cause of reduced gastric acid secretion, and it often results in bacterial overgrowth[21-23]. It is presently possible to reduce gastric acid secretion with H_2-RA or PPI. Treatment with PPI[24-26] or H_2-RA[27] induces a clinical state similar to atrophic gastritis with hypochlorhydria and frequently associated with bacterial overgrowth. Recently, there is considerable information on the efficacy of maintenance treatment with reflux esophagitis for up to 1 year[28-30]. As gastric acid plays an important part in the prevention of bacterial colonization of the stomach and the small intestine, reduction of gastric acid secretion by PPI or H_2-RA results in gastric and intestinal bacterial overgrowth[24-27,31,32]. In fact, it has been reported a marked increase in bacterial titers in fasting gastric aspirates from patients receiving H_2-RA[31,33,34]. On the other hand, the results of identification and quantification of microbes in samples from the gastrointestinal tract are significantly influenced by the culture technique[35].

The most direct method for diagnosing bacterial overgrowth is to perform microbiological cultures after obtaining gastric aspirates. Actually, the microbial flora, which is dominated by *Viridans streptococci*, *coaglase negative Staphylococci*, *Haemophilus sp.*[36], *Neisseria spp.*, *Lactobacillus spp.*, *Candida spp.*, and *Aspergillus spp.*[31], has been demonstrated. However, the study of gastrointestinal flora by direct methods is cumbersome, primary due to its inaccessible location. In addition, the results of identification and quantification of microbes in samples from the gastrointestinal tract are significantly influenced by difficulties in accurate tube placement, contamination during insertion, delay between sampling and inoculation of culture media, and inadequate anaerobic isolation techniques. In addition, intubation methods are time-consuming, and uncomfortable. Therefore, breath tests were devised as simple alternatives to these invasive tests.

Breath hydrogen measurement is now used in clinical practice to investigate several disorders, including small intestinal disaccharidase deficiencies, intestinal bacterial overgrowth, and orocecal transit time[1-8]. It is based on the ability of the anaerobic microflora of the colon to ferment carbohydrate that has traveled unabsorbed through the small intestine, and to produce hydrogen. This hydrogen is transported to the lungs and exhaled in the expired breath. Although breath tests, such as measuring fasting or postprandial hydrogen concentrations, are a noninvasive method, avoiding the risk of sampling error, it is unable to identify the site of overgrowth. Then, we attempted to measure intragastric hydrogen and methane concentrations so as to determine the site of bacterial overgrowth and the incidence of fermentation in the stomach.

We previously reported the endoscopic ^{13}C-urea breath test (e-UBT)[37] in which intragastric gas was collected and analyzed. Using the same sample collection method as e-UBT, hydrogen and methane gases, which are produced by hydrogen-producing bacteria, could be detected in the stomach. These values were considered to reflect directly the intragastric fermentation.

To the best of our knowledge, our study is the first investigation to measure directly intragastric hydrogen and methane concentrations. The values of intragastric hydrogen concentrations above 21 ppm and methane above 8 ppm were considered abnormal in this study. When a patient had a high level of hydrogen or methane in one or more samples, the patient was considered to have fermentation. The overall incidence of intragastric fermentation was 15.4% (73/473). There was no difference in the incidence of intragastric fermentation determined by hydrogen or methane concentrations. These results reveal that intragastric fermentation is found in more than 15% of patients without medication even after overnight fasting.

In this study, intragastric methane levels were higher in the postoperative group than in other groups, whereas there was a negligible difference of intragastric hydrogen levels between the postoperative stomach group and other groups, thereby suggesting that previous gastric surgery is more closely correlated to methane-producing bacterial overgrowth in the stomach, compared to hydrogen-producing bacterial overgrowth although the exact mechanism is unknown.

On the other hand, Fried *et al*[26] reported that most of the bacteria identified from the duodenal aspirates belonged to species colonizing the oral cavity and pharynx, suggesting a descending route of colonization. Also, Thompson *et al*[38] indicated that fermentation of ingested carbohydrate by oropharyngeal bacteria could contribute to measure breath hydrogen values soon after meal ingestion. In addition, many investigators have reported that treatment with PPI[23-25] or H_2-RA[26] induces a clinical state similar to atrophic gastritis with hypochlorhydria and a marked increase in bacterial titers in fasting gastric aspirates from patients receiving H_2-RA[18,20,21]. Although Husebye *et al*[36] have reported that fasting hypochlorhydria associated with gastric colonization of microbes belonging to the oro- and nasopharyngeal flora is highly prevalent in healthy old people, atrophic gastritis is the most common cause of reduced gastric acid secretion, and it often results in bacterial overgrowth[21,22]. We previously reported that intestinal metaplasia was detected in 65.4% (358/547) patients with serum IgG antibody to *H pylori*. Therefore, we expected that a discriminative value might be detected between *H pylori*-positive and *H pylori*-negative patients. As shown in Figure 3, none of the mean values were related to *H pylori* infection.

In summary, we measured directly intragastric hydrogen and methane concentrations using endoscopy and found the incidences of hydrogen and methane production in the stomach. In this new method, the intragastric

gas can be easily collected endoscopically and it does not take much time. Hydrogen and methane gases are more frequently detected in the stomach than expected, regardless of the presence of abdominal symptoms. Moreover, previous gastric surgery influences on the production of methane in the fasting stomach and probably the growth of methane-producing bacteria in the upper digestive tract.

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RAPID COMMUNICATION

Seventy-five gram glucose tolerance test to assess carbohydrate malabsorption and small bowel bacterial overgrowth

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Abstract

AIM: To investigate non-invasively the incidence of absorption of carbohydrates in diabetic patients during an oral glucose tolerance test (OGTT) and to determine whether malabsorption may be associated with insulin secretion and insulin resistance.

METHODS: A standard 75-g OGTT was performed in 82 diabetic patients. The patients received 75 g of anhydrous glucose in 225 mL of water after an overnight fasting and breath samples were collected at baseline and up to 120 min after ingestion. Breath hydrogen and methane concentrations were measured. Blood glucose and serum insulin concentrations were measured before ingestion and at 30, 60, 90, 120 min post-ingestion.

RESULTS: When carbohydrate malabsorption was defined as subjects with an increase of at least 10 ppm (parts per million) in hydrogen or methane excretion within a 2-h period, 28 (34%) had carbohydrate malabsorption. According to the result of increased breath test, 21 (75%) patients were classified as small bowel bacterial overgrowth and 7 (25%) as glucose malabsorption. Patients with carbohydrate malabsorption were older and had poor glycemic control as compared with those without carbohydrate malabsorption. The HOMA value, the sum of serum insulin during the test and the Δinsulin/Δglucose ratio were greater in patients with carbohydrate malabsorption.

CONCLUSION: Insulin resistance may be overestimated by using these markers if the patient has carbohydrate malabsorption, or that carbohydrate malabsorption may be present prior to the development of insulin resistance. Hence carbohydrate malabsorption should be taken into account for estimating insulin resistance and β-cell function.

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Key words: 75-g OGTT; Carbohydrate malabsorption; Bacterial overgrowth; Breath test; Insulin resistance

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INTRODUCTION

The oral glucose tolerance test (OGTT) is a widely used procedure in the diagnoses of diabetes and intermediate stages of hyperglycemia. Plasma glucose and insulin responses during this test reflect the ability of pancreatic β-cells to secrete insulin and the sensitivity of tissues to insulin[1]. However, it was reported that 2%-20% of carbohydrates escape small intestinal absorption[2]. Based on this fact, digestion and absorption of carbohydrates may affect the results of OGTT. If the patient had carbohydrate malabsorption, plasma glucose and insulin responses during OGTT should be lower. Furthermore, an interesting observation was the outcome of OGTT yielding a remarkable intra-individual variability[3] and this was explained by the hypothesis that the velocity of the initial phase of glucose emptying from the stomach may depend on the grade of interdigestive antral motor activity at the time of glucose ingestion[4]. Although a many investigators have reported a close association between gastric emptying and insulin secretion, the prevalence of absorption of carbohydrates in diabetic patients has been unknown.

H_2 breath tests have been used to evaluate intestinal transit, bacterial overgrowth, and disaccharidase

deficiency[5-12]. Because bacteria represent the sole source of gut H_2 and CH_4, fasting breath H_2 and CH_4 gases have been used as markers of colonic fermentation[13,14]. As H_2 production increases when a small amount of carbohydrate is supplied to colonic bacteria, the measurement of breath H_2 concentration has been proposed as an indicator of carbohydrate malabsorption[6]. Similarly, breath CH_4 excretion, which reflects an indirect measurement of the metabolism of the anaerobic colonic flora, has been measured[15,16]. Methanogenic bacteria utilize H_2, carbon dioxide (CO_2), and then synthesize CH_4[17]. CH_4 absorbed from the colon reaches the lung and excretes into the breath[18].

The aim of the present study was to investigate non-invasively the incidence of malabsorption of carbohydrates in diabetic patients during OGTT and to determine whether malabsorption may be associated with insulin secretion and insulin resistance.

MATERIALS AND METHODS

Patients

A standard 75-g OGTT was performed in 82 diet-controlled diabetic patients (42 women and 40 men; age range 30-84 years, average 62 years) without abdominal symptoms. Patients treated with alpha-glucosidase inhibitors were excluded from this study. None of the patients had a history of use of PPI, H_2-receptor antagonist, antibiotics, steroids, or nonsteroidal anti-inflammatory drugs for a period of at least six month before the investigation. Patients who had a previous history of partial gastrectomy were also excluded from the study. The study was approved by our Local Ethical Committee.

Procedures

The patients received 75 g of anhydrous glucose in 225 mL of water in the sitting position after an overnight fasting. Breath samples were collected at baseline and at 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 min after ingestion. Breath H_2 and CH_4 concentrations were measured with breath analyzer TGA-2000 (TERAMECS, Kyoto) and expressed in parts per million (ppm). Linear accuracy response range was 2 to 150 ppm. An increase of at least 10 ppm within a 2-h period is indicative of bacterial overgrowth or glucose malabsorption[19]. Venous blood samples were obtained before ingestion and at 30, 60, 90, and 120 min after ingestion and blood glucose and serum insulin concentrations were measured.

Calculations

The homeostasis model assessment (HOMA), fasting serum insulin, the sum of serum insulin during the test, and the Δinsulin/Δglucose ratio were used as indexes of insulin resistance. The HOMA value was calculated as follows: HOMA = fasting plasma glucose (mg/dL) × fasting insulin (pmol/L)/405. The Δinsulin/Δglucose ratio was calculated as follows: (insulin at 30 min - insulin at baseline)/(glucose at 30 min - glucose at baseline).

Statistical analysis

All values were expressed as means ± SD. Comparisons of groups were made using the Mann-Whitney *U* test. A *P* value less than 0.05 was considered statistically significant.

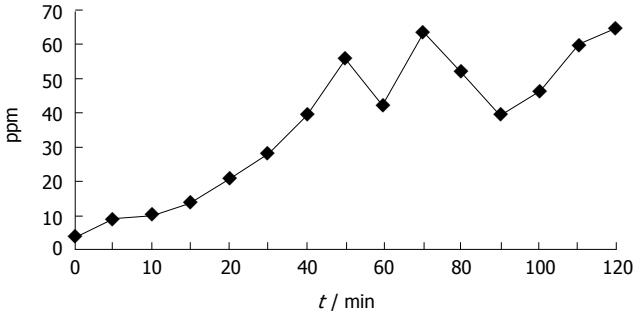


Figure 1 Changes in breath hydrogen concentration during OGTT in the patient with suspicious of small bowel bacterial overgrowth. An increase of H_2 greater than 10 ppm above the baseline was found at 15 min.

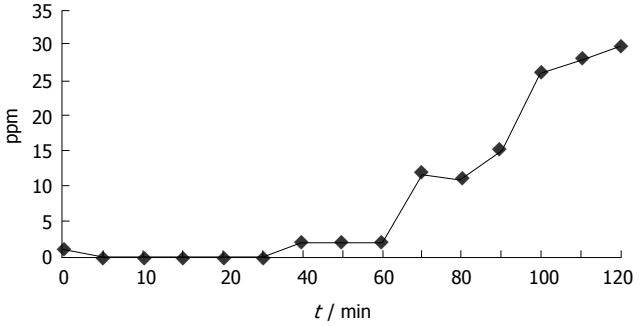


Figure 2 Changes in breath hydrogen concentration during OGTT in the patient with suspicious of carbohydrate malabsorption. Breath H_2 concentration increased gradually from the beginning and reached 10 ppm over the baseline at 70 min and later.

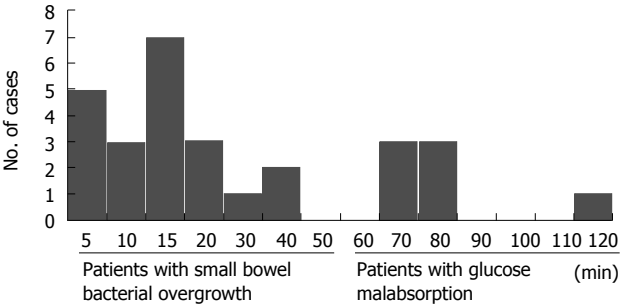


Figure 3 Distribution of time when breath hydrogen and/or methane levels increased more than 10 ppm over baseline.

RESULTS

Incidence of carbohydrate malabsorption

When carbohydrate malabsorption was defined as subjects with an increase of at least 10 ppm within a 2-h period, 34% (28/82) patients had carbohydrate malabsorption. As shown in Figures 1 and 2, small bowel bacterial overgrowth was defined as an increase of H_2 and/or CH_4 greater than 10 ppm above the baseline before the first 40 min, whereas carbohydrate malabsorption without small bowel bacterial overgrowth was defined as an increase of H_2 and/or CH_4 greater than 10 ppm above the baseline at 70 min and later. Of 28 patients with an increase of H_2 and/or CH_4, 21 (75%) patients were classified as small bowel bacterial overgrowth and 7 (25%) as carbohydrate malabsorption (Figure 3). All

Table 1 Characteristics of 82 patients with diabetes mellitus classified according to carbohydrate malabsorption status

	Carbohydrate malabsorption (+)	Carbohydrate malabsorption (-)	*P* value
n	28	54	
Age	63.3 ± 10.3	59.2 ± 9.7	0.34
HbA1c	7.1 ± 1.7	6.4 ± 1.5	0.17
HOMA	2.0 ± 2.1	1.9 ± 1.9	0.22
ΣIR	107.0 ± 95.3	117.0 ± 76.2	0.08
ΔIR/ΔBG	0.18 ± 0.27	0.14 ± 0.21	0.06

28 patients had no abdominal symptom during OGTT.

As shown in Table 1, the values of HbA1c were 7.11% ± 1.70% and 6.43% ± 1.46% in patients with and without carbohydrate malabsorption (including small bowel bacterial overgrowth), respectively. Patients with carbohydrate malabsorption were older and had poor glycemic control when compared with those without carbohydrate malabsorption.

Carbohydrate malabsorption and insulin resistance

The HOMA values in the patients with carbohydrate malabsorption (2.0 ± 2.1) were greater than those without carbohydrate malabsorption (1.94 ± 1.86), but did not reach statistical significance ($P = 0.22$). Similarly, the sum of serum insulin during the test and the Δinsulin/Δglucose ratio were also greater in patients with carbohydrate malabsorption than those without carbohydrate malabsorption (Table 1), but the difference was not significant.

DISCUSSION

Impaired intestinal and gastric motility are frequent findings in diabetic patients[19,20]. However, there is a wide range of symptoms in gastrointestinal motility disorders, and the degree of motility disorders correlate poorly with severity of symptoms. Intestinal peristalsis as well as gastric acid secretion are the most important factors protecting against the small bowel bacterial overgrowth[21]. Although delayed gastrointestinal transit potentially causes bacterial overgrowth, in contrast, a rapid transit may also cause diarrhea due to an increase in intraluminal contents that reach the cecum. It is, therefore, possible that both rapid and delayed gastrointestinal transit cause diarrhea because of bacterial overgrowth or carbohydrate malabsorption. However, patients with bacterial overgrowth may also be asymptomatic[22]. Although gastrointestinal symptoms are present in 50%-70% of diabetic patients, the association between symptoms and bacterial overgrowth in diabetic patients has been unknown.

In the present study, 34% (28/82) patients without abdominal symptoms, including diarrhea, had carbohydrate malabsorption, including 7 patients classified as small bowel bacterial overgrowth. It has been unclear how much of small bowel bacterial overgrowth is opposed to carbohydrate malabsorption. The results of the present study suggest that carbohydrate malabsorption occurs more often in diabetic patients than small bowel bacterial overgrowth.

There is increasing evidence that postprandial hyperglycemia has a major role in the pathogenesis of diabetic macrovascular complications[23,24]. It is widely recognized that postprandial glycemia is potentially dependent on a number of factors, including the rate of carbohydrate entry into the small intestine, small intestinal digestion and absorption, insulin secretion, peripheral insulin sensitivity, and hepatic and muscle glucose metabolism[25]. In addition, postprandial secretion of insulin is prompted as much by the incretin hormones as by entry of glucose into the blood, and the release of incretins is dependent on rates of nutrient entry into the small intestine[26,27]. Our observations confirmed that one third of patients with diet-controlled type 2 diabetes and without abdominal symptoms had carbohydrates malabsorption, which might contribute to postprandial hyperglycemia[25]. Some previous studies also described approximately 35% of the variance in initial postprandial blood glucose concentrations after a 75-g oral glucose load[28,29]. Since patients with carbohydrate malabsorption tended to be older and to have poor glycemic control as compared with those without carbohydrate malabsorption, it is possible that patients with long-standing or poor-controlled diabetes may more often have carbohydrate malabsorption. Conversely, the result and its interpretation of 75-g OGTT might be influenced by carbohydrate malabsorption.

A standard 75-g OGTT has also been used to assess insulin resistance and insulin release. For example, fasting plasma insulin concentrations have been used as an index of insulin resistance, and the 30-min ratio of changes in plasma insulin and glucose has been used as an index of β-cell function[30,31]. Insulin resistance, evaluated by the HOMA value, the sum of serum insulin during the test, and the Δinsulin/Δglucose ratio were greater in patients with carbohydrate malabsorption than those without carbohydrate malabsorption. However, these differences were not significant. This suggests that insulin resistance might be overestimated by using these markers if the patient has carbohydrate malabsorption, or that carbohydrate malabsorption might be present prior to the development of insulin resistance. In conclusion, it is more desirable that carbohydrate malabsorption should be taken into account for estimating insulin resistance and β-cell function.

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RAPID COMMUNICATION

Low serum levels of ghrelin are associated with gallstone disease

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Abstract

AIM: To explore the role of ghrelin in gallstone disease.

METHODS: We carried out a cross-sectional study in 150 subjects, 38 with gallstones (cases) and 112 controls. We also did a real-time PCR-RT study in twenty gallbladder samples each. Body mass index (BMI), serum insulin, ghrelin, and serum lipids were measured. Logistic regression analyses (univariate and multivariate) were conducted to estimate the probability of gallstone disease associated with serum ghrelin concentrations.

RESULTS: Cases were statistically different from controls in gender distribution (P = 0.01), age (53 *vs* 44 yr, P = 0.002), BMI (28 *vs* 25; P = 0.004), and glucose (5.26 *vs* 4.98 mmol/L; P = 0.05). The prevalence of ghrelin serum levels above the third tercile was lower in subjects without metabolic syndrome (P < 0.05). In a multivariate model, we found a protective effect, when ghrelin values were higher than the median value (OR = 0.27, 95%CI 0.09-0.82, P = 0.02). Twenty (20%) gallbladder specimens expressed ghrelin mRNA.

CONCLUSION: Serum ghrelin concentrations are associated with a protective effect of GD.

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Key words: Gallstones; Obesity; Ghrelin; Gallbladder; Gastrointestinal hormones

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INTRODUCTION

Human obesity is associated with many significant comorbidities, including cardiovascular disease, diabetes mellitus, hypertension, stroke, osteoarthritis, and some cancers, gallstone disease (GD), *etc*[1]. GD is exceptionally common in Western populations and is a major source of morbidity in the United States[2] and Latin American countries[3]. A relation between obesity and cholesterol gallstones is well recognized. Obese women are more likely to develop gallstones than are obese men[4]. The bile in obese persons is more lithogenic than is that in nonobese persons, ie, the bile of obese persons has a high ratio of cholesterol to solubilizing lipids (bile acids and phospholipids). This high ratio predisposes to crystallization of cholesterol and gallstone formation. The primary reason for lithogenic bile in obese persons is an increase in total body synthesis of cholesterol[5,6].

Ghrelin is an endogenous ligand of the growth hormone secretagogue receptor and a potent stimulator of growth hormone release in humans[7] Ghrelin is synthesized primarily by the stomach, and in substantially lower amounts by the bowel, pituitary, kidney, placenta, and hypothalamus[8]. In both rats and humans, the ghrelin gene is made up of 4 exons and 3 introns and the precursors contain 117 amino acids (preproghrelin). Ghrelin processing results in different splicing and/or posttranslational mechanisms[9]. Two endogenous growth hormones-releasing peptides have been identified, ghrelin and growth hormone-releasing hormone (GHRH) acts on the GHRH receptor to activate adenylate cyclase and

increase intracellular cAMP, which serves as a second messenger to activate protein kinase A. This indicates that the GHRH receptor is coupled to a G_s subunit. On the other hand, ghrelin acts on the GHS-R and activates phospholipase C to generate IP_3 and diacylglycerol, resulting in an increase of intracellular Ca^{2+}, indicating that the ghrelin receptor is coupled to a G_q subunit[7]

Accumulating evidence suggests that ghrelin contributes to the short- and long-term regulation of body weight as a key element of a complex central signaling network that regulates food intake and energy expenditure[10]; its concentrations increase preprandially and decrease after meals, suggesting a role in meal initiation. Intravenous administration of ghrelin stimulates food intake in humans[11] Ghrelin induces a positive energy balance and induces adiposity in rodents by decreasing fat utilization[12]. Ghrelin concentrations are reported to be decreased in obesity[13] and increased after diet-induced weight loss[14].

The aim of this study was to explore the role of ghrelin in gallstone disease.

MATERIALS AND METHODS

Study population

We conducted a cross-sectional study, an immunohistochemistry study and a PCR-RT study in the check-up unit of the Diagnostic Clinic at the Medica Sur Clinic and Foundation (university hospital with subspecialty care) in Mexico City, Mexico. This hospital provides private care for mainly middle- and high-income individuals from Mexico City and the surrounding metropolitan area. Our sample population was formed from a series of consecutive asymptomatic subjects who were referred to the check-up unit by their companies as an annual requirement, not for symptomatic disease. The study included 150 subjects who agreed to participate, 38 subjects found to have GD (25 women, 13 men) and 112 controls (46 women and 66 men without GD). The study was approved by the Human Subjects Committee at The Medica Sur Clinic and Foundation as conforming to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from all subjects before commencement of the study. All subjects were asked to complete a questionnaire that included demographic and medical variables. The primary demographic and medical variables were gender, age, date of birth, tobacco and alcohol consumption, family history of cardiovascular disease, previous medical conditions, and previous surgery.

Medical exams included an abdominal ultrasonographic study on all subjects as part of the check-up evaluation. Cases were defined as subjects with an ultrasonographic diagnosis of GD (see below), and controls were defined as patients with no such evidence (sensitivity and specificity > 95%)[15].

Gallstone disease

Abdominal ultrasound was performed using a Sonoline Elegra instrument (Siemens Medical System, Germany) with a 3.5 MHz transducer. Ultrasound diagnosis of GD was assessed by the presence of strong intraluminal echoes that were gravity-dependent or that attenuated ultrasound transmission (acoustic shadowing). All ultrasonographic studies were evaluated, by the same radiologist on two different occasions. Subjects with previous cholecystectomies were not included in the study.

Diet history questionnaire

Participants completed a food frequency questionnaire with commonly used portion measures specified. The questionnaire included questions on the frequency and brand of multivitamin and individual vitamin supplements. The answers were subsequently electronically scanned and the daily intake of various nutrients was determined using SNUT software, a program developed by the Instituto Nacional de Salud Pública, Mexico City[16], and appropriate to the Mexican population, in order to estimate dietary energy, protein, carbohydrate, total, saturated, polyunsaturated and monounsaturated fat, vitamin, mineral, and antioxidant intake.

Physical examination

Body weight was measured, in light clothing and without shoes, to the nearest 0.10 kg. Height was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Overweight was defined as a BMI ranging from 25 to 29.9 kg/m^2 and obesity when BMI was $\geqslant$ 30 kg/m^2 Waist circumference (to the nearest 0.1 cm) was measured at the midpoint between the lower border of the rib cage and the iliac crest, and hip circumference was similarly obtained at the widest point between hip and buttock.

Analytical procedures

Insulin concentrations were measured using an immunoenzymometric assay (MEIA; Abbott Diagnostics), with inter- and intra-assay coefficients of variation less than 3%.

Serum glucose in the fasting state was measured in duplicate with an automated analyzer. The coefficient of variation for a single determination was 1.5%. Total cholesterol, high-density lipoprotein cholesterol (HDL-C), and triacylglycerol concentrations were measured by enzymatic colorimetric methods, using CHOL, HDL-C plus (second generation) and TG assays (Roche Diagnostics Co., Indianapolis, IN, USA), respectively. Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using the Friedewald formula[17].

Assessment of insulin resistance (IR) was made using the Homeostasis Model Assessment (HOMA-IR), as originally described by Matthews *et al*[18]: HOMA-IR = fasting insulin (U/L) × fasting glucose (mmol/L)/22.5.

Total serum homocysteine was measured with the fully automated Abbott IMx® homocysteine assay (Abbott Laboratories, Abbott Park, IL, USA). Homocysteine is quantified by the intensity of the polarized fluorescent light measured by the IMx optical assembly. Run-to-run CVs (n = 22) were 2.9, 0.8, and 1.7% at total homocysteine concentrations of 7, 12.5, and 25 μmol/L, respectively.

Serum ghrelin concentrations were determined by radioimmunoassay using RIA kits (Linco Research, St.

Table 1 Demographic, anthropometric, biochemical and diet variables between cases and controls

Variable	Cases n = 38 (median min–max)	Controls n = 112 (median min–max)	P-value[1] Mann–Whitney U test
Age (yr)	52.5 9-80	43.5 18-76	0.002
BMI (kg/m^2)	28.1 17-55	25 19-57	0.004
Body fat (%)	34.1 20-45	27.9 11-47	< 0.0001
Insulin (U/L)	6.2 2-18	5.2 2-51	0.11
HOMA-IR	1.4 0.4-4.8	1.2 0.4-14.1	0.07
Homocysteine (μmol/L)	11.0 5-25	9.0 2-20	0.007
Total cholesterol (mmol/L)	4.99 2.48-8.28	5.07 3-8.51	0.80
HDL-cholesterol (mmol/L)	0.98 0.59-1.97	1.09 0.57-2.02	0.10
LDL-cholesterol (mmol/L)	3.1 1.47-5.43	3.31 0.39-6.28	0.50
Triacylglycerol (mmol/L)	1.6 0.64-4.54	1.42 0.46-3.85	0.18
Energy (kJ)	7624 4743-11417	8323 3043-78803	0.06
Diet proteins (%)	16 12-24	16 10-123	0.93
Diet lipids (%)	31 22-46	34 13-500	0.05
Diet carbohydrates (%)	53 34-64	50 13-68	0.05
Ghrelin (ng/L)	660 129-1267	682 117-1467	0.71
Physical activity (yes) [n (%)]	20 (52.6)	65 (58.0)	0.58[1]
Metabolic syndrome [n (%)]	15 (39.5)	18 (16.1)	0.006[1]

[1] Fisher's exact test (two-tailed).

Charles, MO, USA). The intra- and inter-assay coefficients of variation were both less than 5%.

Metabolic syndrome

Participants having three or more of the following criteria were defined as having the metabolic syndrome[9]. The waist to hip ratio > 0.85 and high body fat were defined according to previous definitions[19].

Ghrelin mRNA expression

Twenty products of cholecystectomy due to symptomatic gallbladder disease were collected prospectively and frozen at -70 ℃ until the simple was completed. Then, mRNA was extracted with a one-step technique using guanidine isotiocianate. Complementary DNA was synthesized and processed in real-time PCR using syber green colorant as detector. A qualitative study was performed using β-actin as control.

Statistical analysis

We established a comparison of continuous variables between cases and controls by means of the non-parametric test U of Mann-Whitney due to the non-normality in some variables. Categorical variables compared between cases and controls were evaluated with Fisher's exact test (two-tailed). In order to estimate, as main effect, the probability to be a case (GD) according to serum ghrelin values, we built a multivariate model in a logistic regression analysis, adjusted for potential confounders. The association measure derived from this analysis is the odds ratio (OR) estimate of the exponential of the regression coefficient. The statistical analysis was performed using SPSS/PC v12.0 Software (SPSS, Chicago, IL, USA).

RESULTS

Complete information was obtained in 38 cases and 112 controls. We observed differences between cases and controls in some variables, particularly in age (cases were older), in anthropometric variables (cases were more overweight, had central obesity and a higher percentage of body fat), and cases had a tendency to have more insulin resistance and higher serum homocysteine values than controls. The median of serum ghrelin values did not show a statistic difference between cases and controls (Table 1).

Table 2 Relationship between metabolic syndrome and serum ghrelin levels in subjects with gallstone disease

Ghrelin terciles	Metabolic syndrome criteria 0	1	2	3	4	5
1st	0	1	7	4	1	0
2nd	1	3	3	0	4	2
3rd	1	5	2	4	0	0

In addition, there was a statistic difference in the gender distribution between cases and controls. Cases had more women by proportion than controls (65.8% *vs* 41.1%, $P = 0.01$), more hypertension (42.1% *vs* 11.5%, $P < 0.0001$) and more metabolic syndrome (39.5% *vs* 16.1%, $P = 0.006$) (Table 1).

When analyze the relationship between metabolic syndrome and ghrelin serum levels (in subjects with gallstone disease), we observe that in those subjects with metabolic syndrome (three or more criteria, according ATPIII) the prevalence of ghrelin serum levels above the third tercile was lower than in subjects without metabolic syndrome (26.66% *vs* 34.78%, $P < 0.05$) (Table 2) Similar analysis was applied for subjects without GD, but any significant difference was founded (data not shown).

When we tested the main effect of ghrelin (divided by the median value), a protective association was observed (OR = 0.27, 95%CI 0.09-0.82, $P = 0.02$), indicating a reduction in the probability of being a case subject if

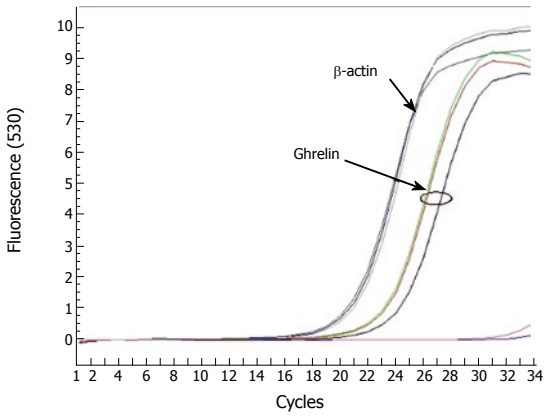


Figure 1 Amplification curves.

the serum ghrelin values are higher than the median, independent of the effect of the control variables: age (yr), gender (males *vs* female), diet lipids (%), homocysteine (μmol/L), BMI (kg/m^2), HOMA-IR, and physical activity (yes *vs* no). All the frozen samples showed ghrelin mRNA (Figure 1).

DISCUSSION

In the present study, we found a statistically significant, negative association of ghrelin serum levels and the prevalence of GD. Obesity is the main risk factor for developing GD and insulin resistance, clinically manifested as the metabolic syndrome. The prevalence of obesity has increased worldwide and is closely associated with an increased morbidity and mortality caused by common diseases of the Western world, such as diabetes, hypertension, coronary heart disease, GD and some cancers[20] Obese individuals are known to have lower ghrelin serum concentrations than healthy controls. In addition, low concentrations of ghrelin are thought to be a consequence of elevated serum levels of insulin and/or leptin, because fasting serum ghrelin concentrations are negatively correlated with fasting serum concentrations of insulin and leptin[13] Recently, ghrelin has been suggested as a regulator of other anorectic cytokines[21] associated with the metabolic syndrome.

Our results could be interpreted as an effect of the negative correlation between ghrelin serum levels and obesity, considering the burden of obesity in GD. However, after controlling for BMI and HOMA-IR, through multivariate analysis, the association remains statistically significant ($P < 0.05$). Even then, the association could arguably be a product of statistical analysis. However, we believed that there are major facts to be considered.

First, ghrelin was originally described both as a "growth hormone-secretagogue" and a "motilin-related peptide". Growth hormone has been associated with gallbladder motility, since hypopituitarism is associated with a form of gallbladder dysmotility that resolves after growth hormone replacement[22] and GD is a frequent complication of long-term octreotide treatment in acromegaly[23].

Second, motilin is a well known small intestine-derived factor in the regulation of gastrointestinal motility in humans involved in receptor-induced gastric motility[24] High motilin concentrations can induce growth hormone releasing effects similar to those of ghrelin and high ghrelin concentrations can induce the gastric prokinetic effect of motilin. Luiking *et al* showed that motilin induces gallbladder emptying in humans[25] and Stolk *et al*[26] observed an impaired motilin-response in fasting cholesterol gallstones patients.

Third, obesity-related insulin resistance could be a determinant factor in the pathophysiology of GD. Scragg *et al*[27] demonstrated higher insulin serum concentration in patients with GD, and an associated increase in the bile cholesterol saturation index[28,29]. In the present study, we observed that cases had lower ghrelin serum levels and higher prevalence of metabolic syndrome, which we have previously reported as a risk factor for GD[30]. Interestingly, Langenberg *et al*[31] reported that lower ghrelin levels were associated with a significantly higher prevalence of the metabolic syndrome, with progressively lower ghrelin levels as the number of metabolic syndrome components increased.

Fourth, We demonstrated the expression of mRNA of ghrelin in frozen tissue, suggesting that the gallbladder is capable of producing ghrelin.

In conclusion, our results suggest that serum ghrelin concentrations are associated with a protective effect of GD. We speculate that this is related with a motilin-like effect of ghrelin on the gallbladder motility. However, further studies are needed to determine the exact role of ghrelin in gallbladder physiology.

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RAPID COMMUNICATION

Ileoscopy in 39 hematochezia patients with normal colonoscopy

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Abstract

AIM: To assess the role of retrograde terminal ileoscopy in hematochezia patients with normal colonoscopy.

METHODS: Between January 1997 and March 2005, 39 hematochezia patients (males 36, females 3, mean age 44.7 years) with a reported normal colonoscopy underwent a repeat colonoscopy. After reaching the cecum, attempt was made to localize the ileocecal valve and intubate the terminal ileum. Any abnormality in the mucosa of the terminal ileum was carefully recorded and biopsies were obtained from suspicious-looking lesions.

RESULTS: During the study period there were 39 patients admitted for hematochezia in whom colonoscopy till cecum did not reveal any abnormality. Full-length colonoscopy till the cecum could be performed in all the patients. The terminal ileum could be intubated in 36 patients. No abnormality was noted in 31 patients. Ileal ulcers were noted in two patients. Nodularity along with ulceration of the ileal mucosa, a Dieulafoy's lesion, and an angiomatous malformation were noted in one patient each. Histological examination of the biopsies obtained from the ulcers revealed typical tuberculous lesion in the patient with nodularity and ulceration. One of the patients had typhoid ulcers and another had non-specific ulcers.

CONCLUSION: Retrograde terminal ileoscopy gives limited but valuable information, in patients with hematochezia and should be attempted in all such patients.

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Key words: Endoscopy; Ileum; Colon; Diagnosis; Tuberculosis; Ulcer; GI bleeding

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INTRODUCTION

Colonoscopy is an established procedure for investigating patients with diseases of the colon and terminal ileum, including patients with prior colonic adenomas or cancer, fecal occult blood loss, a family history of cancer, inflammatory bowel disease, evaluation of iron deficiency anemia and patients with hematochezia[1]. However, although it is expected that endoscopists should be able to intubate the cecum in 90% of attempts[2], in practice this is followed less often than not[3]. On several occasions even a complete colonoscopic examination may not be possible or may not show any abnormality in patients with hematochezia. Moreover, during active bleeding subtle changes in the colon may not be appreciated and sometimes even gross lesions may be missed. Due to this argument and the fact that most of the bleeding will stop by itself, some workers advise that colonoscopy should be performed only after cessation of active bleeding and after proper colonic preparation[4].On the contrary, others prefer to perform colonoscopy as an emergent procedure, so that more lesions are detected early enough for endoscopic therapy to be applied[5, 6].

Terminal ileoscopy is an integral part of complete colonoscopy. Retrograde terminal ileoscopy has been noted to be useful in patients with inflammatory bowel disease, diarrhea, lymphoma, cytomegalovirus-induced ileitis, tuberculosis, portal hypertension and a host of other conditions involving the terminal ileum[7-21]. In a recent study, we have noted that obtaining blind biopsies from even a normal-appearing terminal ileum is useful in patients suspected to have ileocaecal tuberculosis[22]. However, despite being recommended by several authors[7-19, 22], terminal ileoscopy is not routinely performed when colonoscopy is done in all patients undergoing colonoscopy [3]. A more recent study has noted that routine ileoscopy is very useful and easy to perform after proper training has been imparted. However, although stray reports have noted abnormalities in the terminal ileum in hematochezia patients with normal colonoscopy, we are not aware of any study that has dealt with this problem in detail.

MATERIALS AND METHODS

Patients

Between January 1997 and March 2005, colonoscopy with

retrograde ileoscopy was attempted in all patients with hematochezia in whom colonoscopy till the cecum was reported to be normal either during emergency colonoscopy or a routine colonoscopy performed immediately after cessation of bleeding. Upper gastrointestinal (UGI) endoscopy was also performed in these patients but did not reveal any abnormality. Two of these patients had another episode of hematochezia in the past (8 mo and 13 mo before the present hospitalization) and received 4 and 6 units of whole blood transfusion respectively. UGI endoscopy and colonoscopy performed elsewhere did not reveal any abnormality.

Methods

Repeat colonoscopy was performed in our unit. Colonic preparation was performed with either one liter 10% (100 g/L) mannitol solution followed by a WHO oral rehydration solution or a polyethylene glycol-electrolyte-based solution (Peglec, Tablets India, Ltd., Chennai, India). On repeat colonoscopy, a careful search was made for any lesion in the colon. After reaching the cecum, attempt was made to localize the ileocaecal opening and intubate the terminal ileum. Once in the terminal ileum all attempts were made to go as far as possible in the ileum. Any abnormality in the mucosa of the terminal ileum was carefully recorded and biopsies were obtained from suspicious-looking lesions. Biopsies were not obtained from lesions that were likely to be of vascular origin such as ileal varices, spiders, angiomatous malformations and Dieulafoy's lesions.

RESULTS

During the study period there were 39 patients admitted for hematochezia in whom colonoscopy till cecum did not reveal any abnormality. There were 36 males and 3 females. The mean (SD) age of these patients was 44.7 (10.6) years. Of these patients, 34 were admitted to our hospital for hematochezia and the other five were referred from other centers after no lesion could be detected on colonoscopy in these patients. Of the 34 patients admitted to our center, colonoscopy was performed as an emergency procedure in 23 and 11 patients after cessation of the bleeding and bowel preparation. All colonoscopies and ileoscopies were performed by the two senior authors of the paper.

Full-length colonoscopy till the cecum could be performed in all the patients. Colonoscopy did not reveal any abnormality in 38 patients. However, in one of the patients superficial ulcers were seen in the cecum. Despite all efforts, the terminal ileum could not be intubated in three patients. One of the patients had a history of hematochezia 8 months before the present hospitalization.

Of the 36 patients in whom the terminal ileum could be visualized, no abnormality was noted in 31 patients. The other five patients had endoscopic findings on terminal ileoscopy. Nodularity and ulceration of the ileal mucosa were noted in one patient (Figure 1). This patient had superficial ulcers in the cecum too. A Dieulafoy's lesion was noted in one patient (Figure 2). This patient had massive lower gastrointestinal bleeding 13 months prior to the present hospitalization. An UGI endoscopy and colonoscopy at that time did not reveal any abnormality and the patient recovered after conservative management. Another patient had an angiomatous malformation (Figure 3). Ulcers were noted in the terminal ileum in two patients. One of these patients had typhoid fever (Figure 4). In the other patient ulcers were noted only on the ileal side of the ileocecal valve (Figure 5).



Figure 1 Retrograde terminal ileoscopy showing nodularity and ulceration in the ileal mucosa.



Figure 2 Dieulafoy's lesion of the terminal ileum.



Figure 3 Angiomatous malformation seen at ileoscopy.



Figure 4 Ileal ulcers seen at retrograde ileoscopy.



Figure 5 Non-specific ulcers in the terminal ileum.

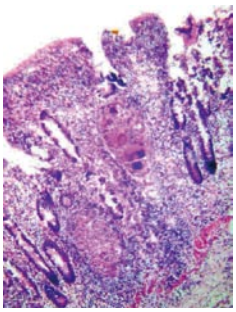


Figure 6 Histological examination of the biopsies obtained from the area of nodularity and ulceration in the terminal ileum showing noncaseating granulomas consisting of Langhans' giant cells and epithelioid cells. Note that a cuff of lymphocytes surrounds the granuloma (HE ×80).

Biopsies obtained from the superficial ulcers in the cecum showed non-specific inflammatory changes. Histological examination of the biopsies obtained from the nodularity and ulcers in the ileum of this patient revealed non-caseating granulomas consisting of Langhans' giant cells and epithelioid cells with a surrounding cuff of lymphocytes (Figure 6), suggestive of tuberculosis. The two other patients with ulcers showed evidence of non-specific inflammation. The patient with typhoid fever was diagnosed to have typhoid ulcer and the other patient to have non-specific ulcer.

DISCUSSION

Colonoscopy remains the procedure of choice for most diseases of the large bowel, including patients with hematochezia[1]. However, the timing of colonoscopy in patients with hematochezia is debatable. While some workers feel that an emergency colonoscopy should be performed in such patients due to the fact that it can detect evanescent findings such as stigmata of recent bleeding or reversible ischemia and can lead to a rapid diagnosis of the cause culminating in its endoscopic management[5, 6], the detractors of urgent colonoscopic examination feel that urgent colonoscopy decreases sensitivity in the presence of active bleeding and that in most cases there is spontaneous cessation of bleeding, even without rendering endoscopic therapy[4]. Others prefer to perform a bleeding scan and angiography in such patients. The advantages of this procedure include the ability to identify the bleeding site whether in the small or in the large bowel, the lack of preparation required for colonoscopy and the ability to perform therapeutic interventions[23]. No matter what the choice of the investigation, an UGI endoscopy must be performed in all such patients, since in about 10% of such patients an UGI source may be responsible for the hematochezia[24]. Although colonoscopy is performed routinely for suspected abnormalities in the colon[1], ileoscopy is not commonly employed in the work-up of patients with ileocolonic abnormalities[3]. This is despite the fact that a number of studies have demonstrated the utility of retrograde ileoscopy during colonoscopic examination of the large bowel[7-22]. A recent study speculated that perceived difficulty of ileal intubation, time constraints, and the expectation of a low diagnostic yield are the likely reasons for the resistance on the part of the endoscopists in attempting retrograde ileoscopy[3].

The results of this study show that a carefully performed colonoscopy that includes retrograde terminal ileoscopy is a very useful tool to diagnose the cause of hematochezia in patients in whom an earlier colonoscopic examination has not revealed any abnormality. Although the yield appeared to be low (13%), the lesions encountered were important in the sense that the diagnosis could be achieved, helping in the treatment of these lesions. In another study, the yield of ileoscopy, performed with an enteroscope in patients with unexplained gastrointestinal bleeding, was noted to be even lower than that noted in the present study. However, in that study the ileal intubation could be achieved in only 39% of patients[25], which is far less than that achieved in most other studies[10, 16, 20-22]. The probable reason for the low ileal intubation rate is the fact that the authors used a more flexible enteroscope instead of a conventional colonoscope for ileoscopy. Moreover, only 10 patients with unexplained gastrointestinal bleeding were enrolled in that study as opposed to those with hematochezia in the present study. It is not clear whether or not these patients had lower gastrointestinal bleeding severe enough to cause hematochezia. The chances of finding a lesion in the terminal ileum was considerably more in our group of patients due to the fact that they had lower gastrointestinal bleeding severe enough to cause hematochezia.

Two of the patients in this series had what is called as obscure GI bleeding[26]. They had an episode of hematochezia in the past and UGI endoscopy as well as colonoscopy did not reveal any abnormality. Unfortunately in one of these patients, despite all attempts, the terminal ileum could not be intubated. However, in the other patient a Dieulafoy's lesion was detected. If ileoscopy was performed with the lesion detected and treated during the previous colonoscopy, the patient would have been spared of a potentially life-threatening episode of hematochezia, suggesting that colonoscopy and ileoscopy should be performed in patients with obscure GI bleeding [26]. We would go a step further and suggest that ileoscopy should be performed while a colonoscopy is performed for all GI bleeding. Waiting for a second episode of bleeding (that might be massive) so as to be labeled as obscure GI bleeding is not warranted. In fact ileoscopy should be an integral part of colonoscopy and all attempts should be made to intubate the terminal ileum during colonoscopy performed for any indication.

Ileoscopy was noted to be useful in one of our patients in diagnosing the cause of the ulcer. In this patient an earlier colonoscopic examination missed superficial ulcers in the cecum. However, histological examination of biopsies obtained from the ulcers in the cecum showed

non-specific changes. If ileoscopy was not performed and biopsies were not obtained from the ileal ulcers, the diagnosis of a curable disease would not have been possible. We have earlier observed such differences in the histological appearance of biopsies obtained from different areas of the colon in patients with colonic tuberculosis[27]. Patients with isolated tuberculous involvement of the terminal ileum have been reported[18].

Except for one patient in whom superficial cecal ulcers were missed on colonoscopy, all the other patients had their disease localized to the terminal ileum. This was because of the design of the study, since patients with lesions found at the first colonoscopy (emergency or elective) were not enrolled. However, in an earlier study, ileoscopy revealed the findings or the diagnosis in 8 of the 57 cases where it was possible and colonoscopic as well as radiological examinations were normal. This group included two patients with lower GI bleeding in whom colonoscopy was normal and ileoscopy revealed typhoid ulcers and NSAID-induced ulcers respectively[16].

The findings noted in this study may not be totally valid in the West, since endoscopists in the developed countries are unlikely to encounter a patient with hematochezia due to typhoid ulcers or tuberculosis. However, with tuberculosis raising its head once again in the developed countries[28-30], such a scenario cannot be totally discounted. The other lesions noted in the present series such as Dieulafoy's lesion, non-specific ulcers and angiomatous malformations are universal diseases and may be seen more commonly.

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RAPID COMMUNICATION

Efficacy of one-day quadruple therapy for *H pylori* infection in Chinese patients

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Abstract

AIM: To compare the efficacies of one-day quadruple therapy and seven-day triple therapy in Chinese patients.

METHODS: Sixty consecutive patients with nonulcer dyspepsia and confirmed *H pylori* infection were randomized to receive either omeprazole 40 mg, amoxycillin 1 g, and furazolidone 100 mg, all twice a day for 7 d or omeprazole 20 mg (at breakfast and dinner), amoxicillin 1 g, furazolidone 200 mg, and colloidal bismuth subcitrate 220 mg four times for only one day. *H pylori* status was determined before and at least 5 weeks after therapy by endoscopy with antral and corpus biopsies for rapid urease test and histology.

RESULTS: *H pylori* eradication was successful in 66.67% (20/30) patients in the 7-d group and in 36.67% (11/30) patients in the 1-day group (P = 0.037). Side effects were induced by the treatment in 13.3% (4/30) patients of each group, but these were all self-limiting, short-lasting, and did not require any specific treatment.

CONCLUSION: The one-day quadruple therapy is less effective than the one-week regimen in curing *H pylori* infection in Chinese patients.

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Key words: *H pylori*; Quadruple therapy; Antibiotics; Gastritis; Side effects

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INTRODUCTION

There is clear evidence that *H pylori* is the main etiologic agent of most gastritis, peptic ulcers and gastric cancer[1,2]. Eradication of infection leads to resolution of the gastritis and to spectacular decreases of ulcer relapses and risk of gastric cancer. Treatment of *H pylori* infection is widely accepted for patients with dyspepsia[3,4]. Several regimens for treating *H pylori* infection have been proposed. One-week triple therapies incorporating a proton pump inhibitor (PPI) and two antibiotics are the most frequently recommended treatment for *H pylori* infection in China. Bacterial susceptibility, cost and patient compliance have been shown to be the important factors in determination of anti-*H pylori* therapy regimen. Sometimes, cost has to be taken into account and may be the first important factor in determination for eradication of *H pylori* for patients from poor rural area or countryside. Availability of 1-d quadruple therapies would therefore be of interest, because they will probably reduce cost and side effects and increase tolerance and compliance. A 1-d high-dose quadruple therapy with a combination of amoxycillin (or tetracycline), metronidazole, a bismuth salt and a proton pump inhibitor has led to promising results with eradication rates of 57%-95%[5-8]. However, Wermeille *et al*[9] indicated that a 1-d administration of PPI-based quadruple therapies yielded significantly lower eradication rates (20%) compared to the 7-d regimens (91%). However, experience is limited and the variant results may come from different patient population. In the present study, we therefore aimed to compare the efficacy of a four-drug 1-d treatment with a three-drug 7-d therapy on of *H pylori* infection in Chinese patients with dyspepsia.

MATERIALS AND METHODS

Patients

From November 2004 to April 2005, consecutive dyspeptic *H pylori*-positive patients referred to our hospital for diagnostic upper gastrointestinal tract endoscopy were enrolled in the study. The patients with *H pylori* infection proven by urease test or histology were included in the study. Exclusion criteria were: (1) age under 18 years or

older than 75; (2) allergy to one of the drugs; (3) inability to attend follow-up; (4) previously failed eradication therapy; (5) treatment with antibiotics during the 4 weeks prior to the study; (6) previous ulcer surgery; and (7) liver or kidney disease, severe cardiac or pulmonary disease, alcoholism, drug abuse, or any other conditions associated with poor patient compliance. Eight patients were eventually excluded from the study after randomization. The remaining 60 patients (35 males and 25 female; mean age 54.3 years, range 19-72 years) were included in the study.

Diagnosis of H pylori infection

Four biopsy specimens, two from the antrum and two from the corpus, were obtained using gastroscopy. Biopsy specimens were stained to assess the presence of *H pylori*. Rapid urease test was considered positive if any color change occurred. Patients were considered as *H pylori*-positive when *H pylori* was positive on either histology and/or rapid urease test.

Therapy

Patients were randomized to receive a 7-d course of omeprazole (40 mg), amoxycillin (1 g), and furazolidone (100 mg), all twice daily with meals, or a 1-d course of omeprazole (20 mg, at breakfast and dinner), amoxycillin (1 g), furazolidone (200 mg), and colloidal bismuth subcitrate 220 mg four times a day. During the treatment period, the patients were asked to refer to their study doctor if they had side effects, or the side effects were recorded by means of a structured clinical interview immediately after the end of therapy.

Assessment of H pylori eradication

The efficacy of treatment was evaluated at least 5 weeks after therapy. Endoscopy was performed and a further four biopsy specimens (two from the antrum and two from the corpus) were obtained to assess the *H pylori* eradication status and histology. A patient was considered to be cured if all two tests were negative at the follow-up endoscopy. To evaluate possible recrudescence, a 14C-urea breath test was performed at least 6 mo after eradication therapy. The Medical Ethics Committee of the institution approved the study design. Informed consent was taken from all patients.

Statistical analysis

Proportions were compared using the chi-squared test. Calculations were performed using the SPSS (10.0) for Windows statistical package.

RESULTS

Sixty patients entered the study. Clinical characteristics of the patients are mentioned in Table 1. There were no significant differences between the 1-d quadruple therapy and 7-d triple therapy groups with respect to age, gender, tobacco and alcohol consumption.

Eradication of H pylori

As shown in Table 2, eradication rate in the 1-d group (36.7%) was significantly lower than that in the 7-d group (66.7%). The study was subsequently stopped. The upper gastrointestinal symptoms had improved in 93.3% (21/30) of patients in the 7-d group and in 90% (15/30) of patients in 1-d group.

Table 1 Characteristics of the treated patients

Treatment	*n*	Male/Female	Mean age (range)	smokers *n* (%)	alcohol users *n* (%)
1-d therapy	30	16/14	38.4 (19-71)	6 (20)	7 (23.3)
7-d therapy	30	19/11	43.3 (18-73)	8 (27)	7 (23.3)

Table 2 Evaluation of *H pylori* eradication regimens

	One-day therapy	Seven-day therapy	*P*
H pylori eradication	36.7% (11/30)	66.7% (28/30)	0.037

Table 3 Side effects associated with *H pylori* treatment

Side effects	One-day therapy	Seven-day therapy
Nausea	2	1
Abdominal pain	1	1
Skin rash	0	1
Dizziness	1	1
Side effect (%)	13.30%	13.30%

Side-effects and compliance

As shown in Table 3, there was no difference between the incidences of side-effects in the two groups. Symptoms were almost always mild and not interfering with daily activities. Compliance was 100% in the 1-d therapy as well as in the 7-d therapy group.

DISCUSSION

The optimal anti-*H pylori* treatment should reach high eradication rates with only few and tolerant side-effects, short duration to assure good compliance and lower costs. Three European studies have first stated an effective one-day quadruple therapy for *H pylori* eradication with cure rates being comparable to one-week standard regimen[5-7]. Recently, Lara *et al*[8] showed more optimal eradication rates (95%) for the 1-d therapy group in the United States patients. One-day therapeutic protocols, to our knowledge, have not been studied in Chinese patients. An important consideration is the significantly lower cost of the 1-d regimen compared to the 7-d regimen of the standard regimen. The aim of our study was to investigate the potential advantages of the 1-d quadruple therapy by comparing to a standard 7-d triple therapy.

Unlike the previous reports[5-8], the eradication rate obtained with the 1-d therapy in our study was significantly lower than that obtained with the standard 7-d therapy. Side-effects were minor and did not interfere their daily

activities in the both therapy groups. Compliance and antibiotic resistance are the major factors contributing to the failure of *H pylori* treatment[9-13]. In our study, the compliance was reached 100% in the 1-d therapy group as well as in 7-d therapy group. However, culture was not performed in this study, so we do not know the status of *H pylori* susceptibility to metronidazole and/or furazolidone; therefore, we cannot exclude the possibility of an antibiotic resistance as the reason for our disappointing results. Nevertheless, to date, resistance to amoxycillin and furazolidone are lower as compared with clarithromycin and metronidazole[14,15]. Smoking and drinking do not appear to be the factors in treatment failure with either one-day quadruple therapy or seven-day triple therapy in Chinese patients.

In conclusion, although a 1-d quadruple therapy is less expensive and has fewer side effects, due to the low efficacy obtained in this study, we do not suggest recommending this regimen to be used in Chinese patients for eradication therapy of *H pylori* infection.

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RAPID COMMUNICATION

Optimal injection volume of epinephrine for endoscopic treatment of peptic ulcer bleeding

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Abstract

AIM: To define the optimal injection volume of epinephrine with high efficacy for hemostasis and low complication rate in patients with actively bleeding ulcers.

METHODS: This prospective, randomized, comparative trial was conducted in a medical center. A total of 228 patients with actively bleeding ulcers (spurting or oozing) were randomly assigned to three groups with 20, 30 and 40 mL endoscopic injections of an 1:10 000 solution of epinephrine. The hemostatic effects and clinical outcomes were compared between the three groups.

RESULTS: There were no significant differences in all background variables between the three groups. Initial hemostasis was achieved in 97.4%, 98.7% and 100% of patients respectively in the 20, 30 and 40 mL epinephrine groups. There were no significant differences in the rate of initial hemostasis between the three groups. The rate of peptic ulcer perforation was significantly higher in the 40 mL epinephrine group than in the 20 and 30 mL epinephrine groups ($P < 0.05$). The rate of recurrent bleeding was significantly higher in the 20 mL epinephrine group (20.3%) than in the 30 (5.3%) and 40 mL (2.8 %) epinephrine groups ($P < 0.01$). There were no significant differences in the rates of surgical intervention, the amount of transfusion requirements, the days of hospitalization, the deaths from bleeding and 30 d mortality between the three groups. The number of patients who developed epigastric pain due to endoscopic injection, was significantly higher in the 40 mL epinephrine group (51/76) than in the 20 (2/76) and 30 mL (5/76) epinephrine groups ($P < 0.001$). Significant elevation of systolic blood pressure after endoscopic injection was observed in the 40 mL epinephrine group ($P < 0.01$). Significant decreasing and normalization of pulse rates after endoscopic injections were observed in the 20 mL and 30 mL epinephrine groups ($P < 0.01$).

CONCLUSION: Injection of 30 mL diluted epinephrine (1:10 000) can effectively prevent recurrent bleeding with a low rate of complications. The optimal injection volume of epinephrine for endoscopic treatment of an actively bleeding ulcer (spurting or oozing) is 30 mL.

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Key words: Endoscopic injection; Epinephrine; Volume; Peptic ulcer bleeding

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INTRODUCTION

Endoscopic injection of epinephrine is considered a highly effective and simple hemostatic technique for non-variceal upper gastrointestinal (GI) bleeding[1-3]. Although the rate of initial hemostasis is high, rebleeding occurs in 6% - 36% of patients[4-7]. Rebleeding after initial endoscopic hemostasis remains an important determinant of poor prognosis in patients with hemorrhaging ulcers[8, 9]. The wide variation in rates of recurrent bleeding may result from the different volumes (2-30 mL) of epinephrine injected to achieve hemostasis in various studies[4-7]. Clinical trials in patients with bleeding ulcers disclosed that injection of normal saline solution[10] or distilled water[11] is as effective as absolute alcohol[10] or an epinephrine[11] solution for endoscopic therapy. These findings suggest that tamponade plays a primary role in hemostasis with endoscopic injection therapy, and that injection of larger volumes of epinephrine may be beneficial for preventing recurrent bleeding by prolonging the hemostatic effect of local compression. Lin *et al*[12] found that injection of a large volume (13-20 mL) of 1:1 0000 solution of epinephrine can reduce the rate of recurrent bleeding in patients with high-risk peptic ulcers and is superior to an injection of lesser volumes (5-10 mL) of epinephrine (15.4% *vs* 30.8%). Park *et al*[13] also reported that injection of 35-45 mL of an 1:10 000 solution of epinephrine is more effective in preventing recurrent bleeding from ulcers in the gastric body than injection of 15-25 mL of the same solution (0% *vs* 17.1%). However,

what is the "optimal injection volume" of epinephrine for the prevention of recurrent bleeding is not clear. The aim of our study was to define the optimal injection volume of epinephrine with high efficacy for hemostasis and low complication rate in patients with actively bleeding ulcers.

MATERIALS AND METHODS

Patients

This prospective, randomized, comparative trial was conducted in a medical center. Patients with active bleeding ulcers between January 2003 and June 2005 were admitted to our hospital. Emergency endoscopies were performed in the first day of admission, patients were enrolled in this study if the endoscopy revealed an ulcer with active bleeding defined by the Forrest classification[14] as stage Ia (spurting bleeding) or stage Ib (oozing). Patients were excluded if they were under 18 years of age, pregnant, unable or unwilling to give written informed consent, on anticoagulation therapy, or had a bleeding tendency (platelet count < 20 000/mm^3, prothrombin time >2.0 by international normalized ratio [INR], and activated partial thromboplastin time > 60 s), bleeding gastric malignancy and multiple potential sources of bleeding. Possible complications of endoscopic treatment were discussed with patients and their relatives, and written informed consent was obtained before entry into the trial. The protocol was approved by the Clinical Research Committee of our hospital. Clinical characteristics, including age, gender, use of non-steroidal anti-inflammatory drugs (NSAID), blood pressure, pulse rate, comorbid diseases, coagulopathy (platelet count 20 000/mm^3-50 000/mm^3, prothrombin time 1.3-2.0 by INR), and Hb level were recorded. Shock was defined as systolic pressure less than 100 mmHg and pulse rate greater than 100 beats/min accompanying cold skin, sweating, pallor, and oliguria. Endoscopic characteristics, including type of stigma, ulcer size and location were recorded. One biopsy was obtained from the gastric antrum and one from the gastric body after cessation of bleeding. The presence of *helicobacter pylori* infection was assessed with a rapid urease test.

Methods

Patients were randomly assigned to three groups with 20, 30 and 40 mL endoscopic injections of an 1:10 000 solution of epinephrine. Randomization was performed by using a computer-generated randomization list and sealed envelopes. A single experienced senior gastroenterologist performed all endoscopic hemostatic treatments. The post-procedure care of the patients was conducted by other physicians. A commercially available endoscope (GIF-XQ240, Olympus Optical Co., Ltd., Tokyo, Japan) and a 4 mm, 23 G injector needle (NM-8L, Olympus) were used. An 1:10 000 solution of epinephrine was injected around the bleeding site (2 mL/injection at 2 mm-3 mm from the point of bleeding) until a total volume of 20, 30 and 40 mL respectively in three groups. Initial hemostasis was defined as endoscopically verified cessation of bleeding for at least 5 min. Pain in the epigastric area that developed during endoscopic injection and lasted for more than one hour after the injection was considered to be induced by the epinephrine injection. Patients who complained of epigastric pain due to endoscopic injection were recorded. Throughout the period of endoscopic injection, electrocardiographic monitoring and pulse oximeter monitoring were used to detect arrhythmia and signs of ischemia or hypoxemia. Patients who continued bleeding, despite an injection of the total volume of epinephrine, underwent additional endoscopic treatment with heater probe thermocoagulation, and were then given emergent surgery if they failed to achieve successful hemostasis. After initial endoscopic hemostasis, all patients received acid suppressive therapy with 40 mg omeprazole, intravenously every 12 h for 3 d, then orally (20 mg/d) for 2 mo. If *Helicobacter pylori* infection was confirmed by the rapid urease test, the patient was treated for 1 wk with a triple drug regimen (omeprazole, amoxicillin and clarithromycin) as soon as possible. Recurrent bleeding was defined as a subsequent ulcer bleeding that occurred after the initial bleeding stopped. "Rebleeding" was suspected if one or more signs of ongoing bleeding were found, including fresh hematemesis, melena or bloody stools, aspiration of fresh blood via nasogastric tube, unstable vital signs, and a reduction of Hb by more than 20 g/L within 24 h after initial hemostasis. Patients with rebleeding were confirmed by endoscopy and treated with a second therapeutic endoscopy (epinephrine injection plus heater probe thermocoagulation), and then underwent surgery if they failed to achieve successful hemostasis. The end points of this study were (a) endoscopic therapy failure, i.e., a combination of persistent bleeding and recurrent bleeding during follow-up or (b) death of the patient.

Statistical analysis

Unless otherwise indicated, values were expressed as mean ± SD. The Student *t* test was used to compare the mean values of continuous variables. The chi-square test with Yates correction for continuity and the Fisher's exact test were used when appropriate to compare the categorical variables. Clinical, laboratory and endoscopic parameters were analysed. All statistical analyses were performed using SPSS statistical package (version 11.0) on a personal computer. A p value less than 0.05 was considered statistically significant. Any frequency of death was considered as an adverse outcome. Therefore, if a death occurred, the cause was determined. If death was due to recurrent bleeding, a decision of whether to stop the study early was made. Clinical outcomes compared between the three groups were initial hemostatic rate, recurrent bleeding rate, perforated peptic ulcer, need for emergency operation, transfusion requirements, bleeding-related deaths, 30-day mortality, and admission duration.

RESULTS

A total of 284 patients with actively bleeding ulcers (spurting or oozing) were enrolled in the study. Of these, 56 were excluded because of the absence of informed consent, bleeding tendency, gastric malignancy, or multiple potential bleeding sources at endoscopy. There were 228 patients who were included in the study, 76 in each group. Clinical and endoscopic data obtained for patients at study

Table 1 Clinical and endoscopic variables of patients at study entry (n = 76, mean ± SD)

	Epinephrine (20 mL) n (%)	Epinephrine (30 mL) n (%)	Epinephrine (40 mL) n (%)
Age (yr)	61.5 ± 12.8	60.2 ± 13.3	62.3 ± 12.5
Gender (M/F)	56/20	53/23	55/21
NSAID ingestion	26 (34.2)	24 (31.6)	27 (35.5)
Shock	20 (26.3)	23 (30.3)	21 (27.6)
Comorbid illness	43 (56.6)	41 (53.9)	45 (59.2)
Hb level (g/dL)	7.2 ± 2.8	7.5 ± 2.6	7.6 ± 2.7
Coagulopathy	12 (15.8)	10 (13.2)	13 (17.1)
Positive *H pylori*	44 (57.9)	43 (56.6)	47 (61.8)
Forrest class			
Ia	18 (23.7)	20 (26.3)	19 (25.0)
Ib	58 (76.3)	56 (73.7)	57 (75.0)
Ulcer size			
⩾2 cm	20 (26.3)	22 (28.9)	23 (30.3)
<2 cm	56 (73.7)	54 (71.1)	53 (69.7)
Location of ulcer			
Stomach	41 (53.9)	40 (52.6)	44 (57.9)
Duodenum	32 (42.1)	34 (44.7)	30 (39.5)
Stoma	3 (3.9)	2 (2.6)	2 (2.6)

NSAID: non-steroid anti-inflammatory drug. $P > 0.05$ for all variables between the three groups.

Table 2 Clinical outcomes of endoscopic injection therapy

	Epinephrine (20 mL) n (%)	Epinephrine (30 mL) n (%)	Epinephrine (40 mL) n (%)
Initial hemostasis	74 (97.4)	75 (98.7)	76 (100)
Recurrent bleeding	15/74 (20.3)[b]	4/75 (5.3)	2/72 (2.8)
Perforated peptic ulcer	0 (0)	0 (0)	4 (5.3)[a]
Surgical intervention	4 (5.3)	2 (2.6)	5 (6.6)
Transfusion requirement (units)	4.7 ± 3.4	4.5 ± 3.2	4.2 ± 3.6
Total hospital stay (days)	10.8 ± 3.3	9.7 ± 3.5	10.2 ± 3.1
Deaths from bleeding	0 (0)	0 (0)	0 (0)
30-d mortality	3 (3.9)	2 (2.6)	4 (5.3)
No. of patients that felt pain'	2 (2.6)	5 (6.6)	51 (67.1)[d]

[a]$P < 0.05$ *vs* the 20 mL and 30 mL epinephrine groups; [b]$P < 0.01$ *vs* the 30 mL and 40 mL epinephrine groups; [d]$P < 0.001$ *vs* the 20 mL and 30 mL epinephrine groups;' Epigastric pain induced by endoscopic injection of epinephrine.

entry are outlined in Table 1. There were no significant differences in age, gender, NSAID ingestion, shock, comorbid diseases, Hb level, coagulopathy, positive H pylori status, bleeding stigma, ulcer size or ulcer location between the three groups. The 3 groups were well matched for factors that could potentially affect outcomes (Table 1).

Initial hemostasis was achieved in 74 of 76 patients (97.4%) in the 20 mL epinephrine group, 75 of 76 patients (98.7%) in the 30 mL epinephrine group and 76 of 76 patients (100%) in the 40 mL epinephrine group. There were no significant differences in the rate of initial hemostasis between the three groups (Table 2). Three patients with spurting bleeding from gastric ulcers, two in the 20 mL epinephrine group and one in the 30 mL epinephrine group, failed to achieve initial hemostasis after injection of 20 mL or 30 mL of the 1:10 000 solution of epinephrine. Bleeding was controlled in all patients by additional heater probe thermocoagulation. Four patients in the 40 mL epinephrine group developed perforation of peptic ulcers and underwent emergent operations within 24 h after endoscopic injection. The rate of peptic ulcer perforation was significantly higher in the 40 mL epinephrine group than in the 20 mL and 30 mL epinephrine groups ($P < 0.05$, Table 2). The rate of recurrent bleeding was 20.3% (15/74) in the 20 mL epinephrine group, 5.3% (4/75) in the 30 mL epinephrine group and 2.8 % (2/72) in the 40 mL epinephrine group. The rate of recurrent bleeding was significantly higher in the 20 mL epinephrine group than in the 30 and 40 mL epinephrine groups ($P < 0.01$, Table 2). Fifteen patients in the 20 mL epinephrine group had recurrent bleeding. Bleeding was controlled in 11 patients by epinephrine injection plus heater probe thermocoagulation, and in 4 by surgical intervention. Four patients in the 30 mL epinephrine group had recurrent bleeding. Bleeding was controlled in 2 patients by epinephrine injection plus heater probe thermocoagulation, and in 2 by surgical intervention. Two patients in the 40 mL epinephrine group had recurrent bleeding. Bleeding was controlled in one patient by epinephrine injection plus heater probe thermocoagulation, and in the other by surgical intervention. The rate of 30-d mortality was 3.9% (3/76), 2.6% (2/76) and 5.3% (4/76) in the 20, 30 and 40 mL epinephrine groups respectively. The causes of death included severe pneumonia with respiration failure in 3 patients, terminal lung cancer in 1 patient, advanced hepatoma with hepatic failure in 3 patients, advanced colon cancer with sepsis in 1 patient and uremia in 1 patient. There was no death due to peptic ulcer bleeding in the three groups. There were no significant differences in the rates of surgical intervention, the amount of transfusion requirements, the days of hospitalization, the deaths due to bleeding and 30-d mortality between the three groups (Table 2). The number of patients who complained of epigastric pain during the procedure of endoscopic injection, was significantly higher in the 40 mL epinephrine group (51/76) than in the 20 mL (2/76) and 30 mL (5/76) epinephrine groups ($P < 0.001$, Table 2).

There was no procedure-related cardiovascular or respiratory complication in three groups. Electrocardiographic monitoring did not record any cardiac arrhythmia, and no abrupt change occurred in oxygen saturation. However, significant elevation of systolic blood pressure after endoscopic injection was observed in the 40 mL epinephrine group ($P < 0.01$), while there were no significant changes in systolic blood pressure after endoscopic injection in the 20 and 30 mL epinephrine groups (Table 3). Significant decreasing and normalization of pulse rates after endoscopic injections were also observed in the 20 and

Table 3 Changes in systolic blood pressure and pulse before and after endoscopic injection therapy (n = 76, mean ± SD)

	Epinephrine (20 mL) n (%)	Epinephrine (30 mL) n (%)	Epinephrine (40 mL) n (%)
Pressure (mmHg)			
Before treatment	108.2 ± 31.8	109.3 ± 29.3	106.2 ± 30.7[b]
After treatment	110.3 ± 21.9	112.7 ± 22.5	129.3 ± 25.8[b]
Pulse (/min)			
Before treatment	96.5 ± 14.7[d]	98.2 ± 15.3[d]	96.7 ± 14.3
After treatment	84.8 ± 12.8[d]	86.3 ± 12.5[d]	101.4 ± 15.2

[b]$P < 0.01$ (paired t-test) in the 40 mL epinephrine group; [d]$P < 0.01$ (paired t-test) in the 20 mL and 30 mL epinephrine groups.

30 mL epinephrine groups ($P < 0.01$) as compared to the 40 mL epinephrine group (Table 3).

DISCUSSION

Peptic ulcer bleeding remains a disease with a considerable morbidity and mortality. The mortality rate is high especially for patients with spurting or oozing bleeding[2, 3, 15]. Endoscopic injection of epinephrine, which is the most widely used method, can significantly reduce rates of further bleeding in non-variceal upper GI[1-3]. Although high rates of initial hemostasis have been obtained, rebleeding occurs in 6% to 36% of patients[4-7]. The wide variation in rates of recurrent bleeding may result from the different clinical features and endoscopic findings between enrolled patients and also the different volumes (2-30 mL) of epinephrine injected to achieve hemostasis in various studies[4-7]. Some factors may be associated with the rate of recurrent bleeding in actively bleeding ulcers after endoscopic injection, including age, shock, comorbid illness, ulcer size, ulcer location and stigmata of hemorrhage[2, 9, 16], which were similar between the 3 groups in the present study, suggesting that the outcomes of endoscopic therapy are unlikely influenced by these factors.

A number of agents have been used to achieve hemostasis in endoscopic therapy. However, what is the best solution for the prevention of recurrent bleeding remains unclear. Lai *et al*[11] demonstrated that a local tamponade with distilled water is as effective as epinephrine solution (11.3 ± 4.7 mL) in endoscopic injection therapy. Lin *et al*[10] have also disclosed similar hemostatic effects of injection with normal saline solution, 3% NaCl solution, 50% glucose water, and pure alcohol. These studies suggested local tamponade is the major effect on sustained hemostasis. Although the mechanisms of hemostasis are different among various agents, clinical studies comparing various agents have demonstrated that no single solution is superior to the others[2, 10, 11]. Laine and Estrada[17] found that local tamponade with normal saline (30 ± 3 mL) solution is less effective than thermal therapy. However, Chung *et al* [18] found that epinephrine injection (1.5 to 19.5 mL) and thermocoagulation have comparable rebleeding rates, indicating that epinephrine injection is technically easier to perform and has a higher initial hemostatic rate. Although the most important mechanism underlying hemostasis in response to epinephrine solution is local tamponade, which is similar to normal saline solution, epinephrine solution offers additional hemostatic effects, including vasoconstriction and platelet aggregation[19]. In a patient with massive active bleeding, it is often difficult to achieve hemostasis with the application of contact devices or laser emergently when visualization of the bleeding site is unsatisfactory. In addition, these methods may not be available in rural hospitals. In our study, the initial hemostatic rate in the 20 mL epinephrine group (97.4%) was as high as that in the 30 mL (98.7%) and 40 mL (100%) epinephrine groups, suggesting that epinephrine solution can be used as a first-line injection solution and hemostatic method for the initial arrest of active bleeding.

Because epinephrine produces vessel compression, vasoconstriction, and platelet aggregation, but not induce vessel thrombosis, the addition of sclerosing agents such as ethanol, ethalonamie or polidocanol would be advantageous in theory. However, clinical trials have not definitively demonstrated a benefit for this combination therapy[6, 20-22]. Likewise, the addition of thrombin or fibrin sealant also dose not reduce the risk of rebleeding[23, 24]. Lin *et al* [12] found that injection of a large volume (13-20 mL) of epinephrine can reduce the rate of recurrent bleeding in patients with high-risk peptic ulcer and is superior to injection of lesser volumes (5-10 mL) of epinephrine (15.4% *vs* 30.8%). Park *et al* [13] also reported that injection of 35-45 mL of an 1:10 000 solution of epinephrine is more effective in preventing recurrent bleeding from ulcers in the gastric body than an injection of 15-25 mL of the same solution (0% *vs* 17.1%). These findings suggest that a continuous injection of larger volumes of epinephrine even after the bleeding stopped is beneficial for preventing recurrent bleeding by prolonging the hemostatic effect of local tamponade, vasoconstriction, and platelet aggregation. However, what is the optimal volume of epinephrine that can achieve permanent hemostasis still remains unknown. In our study, the rate of recurrent bleeding was significantly higher in the 20 mL epinephrine group than in the 30 mL and 40 mL epinephrine groups ($P < 0.01$), while there was no significant difference in the rate of recurrent bleeding between these two groups. The studies of Lin *et al*[12] and Park *et al*[13] enrolled peptic ulcers with spurting, oozing or non-bleeding visible vessels, and found that only 48.7% (38/78) and 52.8% (19/36) of ulcers are active bleeders (spurting or oozing) in the larger-volume injection group respectively. Because our study only enrolled patients with active bleeding (spurting or oozing), the rates of recurrent bleeding in the 30 and 40 mL epinephrine groups were higher than those reported by Park *et al*[13]. However, with a larger injection volume, the rebleeding rate in our study was lower than that reported by Lin *et al*[12] Our study disclosed that an injection of 30 mL epinephrine was an optimal volume for sustained hemostasis and equally beneficial for preventing recurrent bleeding as a 40 mL epinephrine injection.

Sung *et al*[25] found that immediately after the endoscopic injection of a small volume (3-11 mL) of 1:10 000 epinephrine solution, the plasma epinephrine level is increased by 4-5 times above the basal level. In addition, hypertensive emergency and ventricular tachycardia after epinephrine injection have been reported[26]. In our study,

significant elevation of systolic blood pressure after endoscopic injection was observed in the 40 mL epinephrine group ($P < 0.01$), while there were no significant changes in systolic blood pressure after endoscopic injection in the 20 and 30 mL epinephrine groups. Significant decreasing and normalization of pulse rates after endoscopic injections were also observed in the 20 and 30 mL epinephrine groups ($P < 0.01$) as compared to the 40 mL epinephrine group. Therefore, the potential risk of inducing a hypertensive crisis and arrhythmia in patients with underlying cardiovascular diseases by using 40 mL of epinephrine as a hemostatic solution should not be overlooked. Although it has been confirmed that endoscopic injection of epinephrine is safe [6, 20, 23], the injection volume of epinephrine should not be unlimited, because the systemic absorption of epinephrine does occur during endoscopic injection. For large-volume epinephrine injection, electrocardiographic and pulse oximeter monitoring should be used to detect arrhythmias and signs of ischemia or hypoxemia. Our study demonstrated that injection of 30 mL epinephrine was safer than injection of 40 mL epinephrine concerning the potential risk of inducing a cardiovascular event.

The epigastric pain induced by large-volume epinephrine injection is rarely reported in literature. In our study, the number of patients who developed epigastric pain due to endoscopic injection, was significantly higher in the 40 mL epinephrine group (51/76) than in the 20 (2/76) and 30 mL (5/76) epinephrine groups ($P < 0.001$). The epigastric pain induced by large-volume epinephrine injection may be due to tissue ischemia as well as swelling of the ulcer. In addition, injection with a large volume of epinephrine may result in severe tissue necrosis[4, 22, 27] or GI perforation[21, 24, 28]. Perforation of duodenal ulcer in a patient injected a large-volume (35 mL) epinephrine has been reported by Park *et al*[13]. In our study, the rate of peptic ulcer perforation was significantly higher in the 40 mL epinephrine group than in the 20 and 30 mL epinephrine groups ($P < 0.05$). Among the four patients who developed perforation of peptic ulcers, three had deep duodenal ulcers (two with spurting bleeding and one with oozing), the other one had spurting bleeding from a deep and big ulcer (4 cm) in the antrum of the stomach. The size of all the perforated ulcers is greater than 2 cm before endoscopic injection. The cause of perforation may be due to severe tissue necrosis and prolonged inflation in the limited space of the duodenal bulb during the endoscopic procedure. Our study revealed that injection with 30 mL epinephrine was safer than 40 mL epinephrine concerning the potential risk of inducing severe tissue necrosis or peptic ulcer perforation.

In conclusion, diluted epinephrine (1:10 000) solution is good as a first-line injection solution for arrest of active bleeding with a high initial hemostatic rate. Injection of 30 mL epinephrine is optimal for sustained hemostasis with a low rebleeding rate. A 30 mL epinephrine injection is safer than a 40 mL epinephrine injection concerning the potential risk of inducing cardiovascular event and severe tissue necrosis or peptic ulcer perforation. Injection of 30 mL diluted epinephrine (1:10 000) can effectively prevent recurrent bleeding with a low rate of complications. The optimal injection volume of epinephrine for endoscopic treatment of an actively bleeding ulcer (spurting or oozing) is 30 mL.

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RAPID COMMUNICATION

Conversion to sirolimus immunosuppression in liver transplantation recipients with hepatocellular carcinoma: Report of an initial experience

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Abstract

AIM: To report a retrospective analysis of preliminary results of 36 patients who received sirolimus (SRL, Rapamune®, rapamycin) in a consecutive cohort of 248 liver allograft recipients.

METHODS: Thirty-six liver transplant patients with hepatocellular carcinoma (HCC) who were switched to SRL-based immunosuppression therapy from tacrolimus were enrolled in this study. The patients who were diagnosed as advanced HCC before orthotopic liver transplantation (OLT) were divided into group A (n = 11), those who were found to have HCC recurrence and/or metastasis after OLT were assigned to group B (n = 18), and those who developed renal insufficiency caused by calcineurin inhibitor (CNI) were assigned to group C (n = 7) after OLT.

RESULTS: The patients were followed up for a median of 10.4 mo (range, 3.8-19.1 mo) after conversion to SRL therapy and 12.3 mo (range, 5.1-34.4 mo) after OLT. Three patients developed mild acute cellular rejection 2 wk after initiating SRL therapy, which was fully reversed after prednisolone pulse therapy. In group A, only 1 patient was found to have HCC recurrence and metastasis 12 mo after OLT. In group B, 66.7% (12/18) patients (2 with progressive tumor, 7 with stable tumor and 3 without tumor) were still alive due to conversing to SRL and/or resection for HCC recurrence at the end of a median follow-up of 6.8 mo post conversion and 10.7 mo post-transplant. In group C, no HCC recurrence was demonstrated in 7 patients, and renal function became normal after SRL therapy. Thrombocytopenia (n = 2), anemia (n = 8), and oral aphthous ulcers (n = 7) found in our cohort were easily manageable.

CONCLUSION: The conversion to SRL-based immunosuppression may inhibit the recurrence and metastasis of HCC and improve CNI-induced renal insufficiency in OLT patients with HCC.

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Key Words: Sirolimus; Orthotopic liver transplantation; Hepatocellular carcinoma

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INTRODUCTION

Sirolimus (SRL, Rapamune®, rapamycin) is a macrocyclic triene antibiotic that was initially found to have antifungal properties, and may act as a primary immune suppressant or antitumor agent. Unlike tacrolimus and cyclosporine, SRL itself does not reduce glomerular filtration rate (GFR) or nephrotoxicity[1]. Liver transplant patients suffering from calcineurin inhibitor (CNI)-related renal insufficiency could be converted to SRL-based monotherapy or combination therapy[2]. SRL has potent antitumor activity *in vitro* and *in vivo*[3-5]. This property may offer a new approach to inhibiting the recurrence and metastasis of hepatocellular carcinoma (HCC) after orthotopic liver transplantation (OLT). Considering these two favorable effects of SRL, we added SRL to our immunosuppressive regimens in November 2003. We here report a retrospective analysis of preliminary results of 36 patients who received SRL in a consecutive cohort of 248 liver allograft recipients. The indications for conversion to SRL-based immunosuppressive regimen with reduction of CNIs included: patients with

advanced HCC before OLT, HCC recurrence or metastasis after OLT, and CNI- induced nephrotoxicity.

MATERIALS AND METHODS

Patients

Between April 2001 and January 2004, a total of 248 patients underwent OLT in our hospital. Among them, 36 (28 men, 8 women) received treatment with SRL. The indication for OLT was HCC combined with hepatitis B-related cirrhosis. These 36 patients were divided into 3 groups: group A: patients with preoperative advanced HCC (n = 11), group B: patients with HCC recurrence and metastasis after OLT (n = 18), group C: patients with CNI-induced renal insufficiency (n = 7). Advanced HCC was defined as stage III-IV according to the pathological TNM classification[6]. Patients were eligible for SRL treatment if the serum creatinine level was ⩾120 μmol/L (normal range, 40-120 μmol/L) on three occasions after exclusion of other causes of nephrotoxicity and after minimization of CNI levels without graft rejection[2]. The study protocol was approved by the Research Ethics Committee of Zhongshan Hospital, and informed consent was obtained from all participants. Patients were followed up for a median of 10.4 mo (range, 3.8-19.1 mo) after conversion to SRL therapy and 12.3 mo (range, 5.1-34.4 mo) after OLT. Chest radiography, abdominal ultrasonography, computed tomographic (CT) scanning and serum alpha-fetoprotein (AFP) measurement were performed once a month during the follow-up period. Liver angiography, chest CT scanning and bone scintigraphy were performed when tumor recurrence or metastasis was suspected.

Immunosuppression

Immunosuppression was started during surgery with 1000 mg methylprednisolone, followed by taper, 240 mg to 40 mg a day for over 6 d. Maintenance prednisone at 5-20 mg daily was then used based on the perceived degree of patient debilitation. Tacrolimus, the routine CNI used in our hospital, was orally administered 12 h after OLT at 0.05-0.1 mg/kg every 12 h. The dose was adjusted to reach target levels of 10-15 ng/mL during the first 14 d. Then the levels were maintained within the range of 6-10 ng/mL during the second 14 d, and 5-8 ng/mL thereafter. Mycophenolate mofetil (MMF) (0.25-0.5 mg, *po*, bid) was used for 12 patients when tacrolimus did not reach the target level after addition of tacrolimus.

Tacrolimus dose was reduced to half, and its concentration was maintained at 2-5 ng/mL. MMF was stopped once SRL therapy was initiated. SRL could impair wound healing in transplant recipients[7]. We started to use SRL in our study at least 1 mo after OLT to avoid affecting the wound healing. All the 36 patients were given SRL at the initial loading dose of 3 mg/m^2. SRL doses were then adjusted to achieve steady-state whole-blood level of approximately 5-8 ng/mL thereafter. SRL oral solution (Wyeth-Ayerst Research, Philadelphia, PA, USA) was administered once daily in the morning, following dilution with water or orange juice. The dose was reduced or increased to 0.5 mg/d if the level was higher than 8 ng/mL or lower than 5 ng/mL.

Table 1 Demographic Data of the patients

	Group A (n = 11)	Group B (n = 18)	Group C (n = 7)
Age (mean±SD yr)	46.6 ± 10.6	47.1 ± 7.8	53.6 ± 5.7
Gender (women/men)	4/7	3/15	1/6
Immunosuppression regimen before switch			
Tacrolimus+steroids+MMF	7	4	1
Tacrolimus+steroids	4	14	6
Immunosuppression regimen after switch			
Sirolimus+tacrolimus	8	13	5
Sirolimus+steroids+tacrolimus	3	5	2

MMF: mycophenolate mofetil.

The patients who were suspected to have rejection underwent liver transplant biopsy. Anti-rejection measures included prednisolone pulse therapy for mild rejection or antibody therapy with thymoglobulin for more severe rejection.

Anti-viral drugs

Lamivudine was started at least 1 wk before OLT (100 mg orally, daily) and continued thereafter. Hepatic B immunoglobulin (800 IU/d) was given intramuscularly for 1 wk, followed by 400 IU twice a week and then once a month according to the anti-HBs titer.

Statistical analysis

Overall survival rate was analyzed using the Kaplan-Meier product limit method. The differences in white blood cell count and serum creatinine levels between the two different groups were evaluated by Mann-Whitney U test. All data were analyzed by SPSS 10.0 (SPSS Inc, Chicago, IL), and $P < 0.05$ was considered statistically significant.

RESULTS

Patient demographics are summarized in Table 1. Tumor extension and histopathological evaluation are summarized in Table 2.

In group A, 11 advanced HCC patients (6 with tumor thrombus in the first right branch of portal vein) were switched to SRL in the early stage (range, 1.0-6.0 mo) after OLT. Only 1 patient was found to have tumor recurrence in liver graft and metastasis in bone and lung 12 mo after OLT, and died at 14 mo posttransplant. The other 10 patients survived without tumor during a follow-up period of 3.9-11.5 mo after conversion to SRL therapy and 5.1- 3.5 mo after OLT.

In group B, 18 patients had tumor recurrence and metastasis within 8 mo after OLT, and were conversed to SRL-based immunosuppression. The overall 1-year survival rate was 68.2%, and 66.7% (12/18) patients were still alive after a median follow-up of 6.8 mo (range, 3.8-14.2 mo) post conversion and 10.7 mo (range, 5.6-20.1 mo)

Table 2 Tumor status at the time of transplantation

	Group A (n = 11)	Group B (n = 18)	Group C (n = 7)
Stage			
I	0	0	4
II	0	0	3
III	4	9	0
IV	7	9	0
Grade (WHO)			
I	1	0	4
II	6	10	3
III	4	8	0
PVTT			
Yes	6	6	0
No	5	12	7

Stage according to pTNM classification[6]; Grade according to WHO definition (grade I: well-differentiated; grade II: moderately-differentiated; grade III, poorly-differentiated); PVTT: portal vein tumor thrombus.

posttransplant. Four patients who had a single nodule of lung metastasis after transplantation underwent local lung resection. After resection, 2 patients were found to have multiple metastases in the lung again and received bronchial arteriographic embolization, and survived for 5 mo and 9 mo with tumor progression. The other 2 patients remained tumor free at 3 mo and 10 mo after resection for lung metastasis. Another patient who was found to have bone (transverse process of the twelfth thoracic vertebra) metastasis 1 mo after OLT underwent metastasis resection and local radiotherapy. She survived without tumor for 11 mo after conversion and 12 mo after OLT. The other 13 patients had multiple organ (liver and lung) recurrence and metastasis posttransplant, and could not undergo surgical ablation. While no additional therapy beyond SRL was given, 53.8% patients (7/13) were still alive, their tumors remained stable in size after SRL, and 46.2% patients (6 /13) died due to the progression of tumor.

In group C, the conversing SRL time for patients with CNI-induced renal insufficiency CNI was different (range, 1.5-17 mo). No tumor recurrence was demonstrated in the 7 cases. Improvement in renal function was found in 7 patients with their serum creatinine levels returned to normal within 60 d after SRL (154.0 ± 36.2 μmol/L *vs* 83.6 ± 11.4 μmol/L, $P < 0.05$). The most significant improvement was achieved during the first 30 d.

In all the 36 patients, only 3 patients developed biopsy-proven mild acute cellular rejection (Banff criteria rejection activity index of 3-4) 2 wk after initiating SRL therapy, which was fully reversed with prednisolone pulse therapy. All the patients were HBsAg negative and HBsAb positive after OLT after receiving lamivudine and hepatic B immunoglobulin treatment. There was no significant decrease in white blood cell count (6137 ± 1974 c/μL *vs* 5725 ± 1642 c/μL, $P > 0.05$). Eight patients developed anemia (hemoglobin < 10 g/L), and 5 received with red blood cell transfusion, 3 had percutaneous injection of recombinant erythropoietin. Platelet count decreased significantly as compared with baseline in 2 patients (from 125.1×10^9/L to 43.3×10^9/L and from 103.6×10^9/L to 51.2×10^9/L, respectively). The other side effect observed was oral aphthous ulcer (7/36). The symptoms of oral aphthous ulcer improved with a dose reduction of SRL in 2 patients. Relief therapies included antiviral and topical anesthetics in monotherapy or combined therapy in 5 patients. No incidence of hepatic artery thrombosis, delay in wound healing or other allograft dysfunction was found. No other serious hematologic or biochemical changes were observed in this study.

DISCUSSION

The use of SRL in chemotherapy or prevention of tumor proliferation is intriguing[8, 9]. The immunosuppressive and antitumor effects of SRL share a common mechanism of action. SRL inhibits the mammalian target of SRL (mTOR), which prevents acute graft rejection mediated by interleukin-2 and blocks other cytokine signal transduction, thus directly inhibiting tumor cell proliferation[9]. The effects are supported by clinical results with SRL in organ transplantation. Kneteman *et al*[5] followed up 21 patients with HCC beyond Milan criteria who underwent OLT and were treated with a SRL-based immunosuppressive protocol. The 1- and 4-year survival rate was 90.5% and 82.9%, respectively. Only 4 patients were found to have tumor recurrences at a median time of 17 mo postplantation, and the median postrecurrence survival time was 15.5 mo. Compared with the survival rate reported by Yao *et al*[10], our initial experience also suggests that SRL therapy has beneficial effects on HCC patient survival. In group A, only 1 patient died at month 14 posttransplant. The other 10 patients acquired tumor-free survival during a follow-up period of 3.9-11.5 mo after conversion and 5.1-13.5 mo after OLT. In group B, the 1-year survival rate was 68.2% and 66.7% (12/18) patients were still alive (2 with progressive tumor, 7 with stable tumor, and 3 without tumor) after SRL therapy (5 patients had resection for HCC recurrence) after a median follow-up of 6.8 mo (range, 3.8-14.2 mo) post conversion and 10.7 mo (range, 5.6-20.1 mo) posttransplant.

Suppression of the immune system by antirejection therapy has long been linked to increased rates of cancer in transplant recipients. Advances in basic medical sciences have strengthened the association with CNI and increased risk of malignant disease recurrence[11, 12]. Hojo *et al*[12] reported that cyclosporine transforms a noninvasive lung cancer cell line into an invasive phenotype. Vivarelli *et al*[13] reported that patients with HCC receiving a high dosage of cyclosporine during post-liver transplant 3 mo to 12 mo experience a significantly lower recurrence-free survival. These results indicate that cyclosporine can exacerbate tumor growth in patients with malignant tumor[14]. Schumacher *et al*[15] recently reported that *in vitro* suppression of human hepatoma cells by SRL, in contrast to tacrolimus, inhibits hepatoma growth. Our immunosuppressive protocol was planned to minimize the potential impact of steroids and CNIs on HCC recurrence, and to allow for benefits of the previously-reported antitumor

activity of SRL[16]. In group A, only 1 patient who had a tumor (14 cm in diameter) with tumor thrombus in the main branch of portal vein in the explant liver, was found to have recurrence and metastasis 12 mo after OLT. The other 10 advanced HCC patients including 5 with tumor thrombus in the main branch of portal vein have survived without tumor recurrence and metastasis till now (range, 5.1-13.5 mo after OLT). While in group B, 18 advanced HCC patients were found to have intrahepatic recurrence or extrahepatic metastases (mainly in lung) within 8 mo after OLT on maintenance immunosuppression with tacrolimus and steroids. We have also made a retrospective analysis of 40 patients with advanced HCC, including pTNM stage III in 21 patients, and pTNM stage IV in 19 patients, and 9 patients with tumor thrombus in the main branch of portal vein, who underwent OLT before adding SRL to our immunosuppressive regimens. HCC recurrence and metastasis were observed in 11 patients after OLT (11/40, recurrence rate 27.5%), which occurred within 8 mo[17]. As such, recurrence time seemed to be later (≤8 mo *vs* 12 mo) in patients with SRL-based immunosuppression. The difference may be most probably ascribed to the antitumor effect of SRL. However, our study is limited in terms of the number of patients and time of follow-up to properly define the effect of antitumor. Nonetheless, SRL offers a new and promising approach to the prevention or management of posttransplant recurrence and metastasis of HCC. The immunosuppressive efficacy and antitumor activity of SRL may confer a unique for the long-term management of OLT for HCC.

In many open-label studies on liver transplant recipients using SRL as part of a primary immunosuppressive regimen, the occurrence of acute cellular rejection is relatively low. Watson *et al*[18] reported the none of their patients on triple-therapy experiences rejection. Similarly, McAlister *et al*[19] reported that rejection is observed in only 14% patients, which is approximately 50% lower than that in historic patients with conventional immunosuppression. During the median follow-up period of 10.4 mo after conversion to SRL therapy in our cohort, only 3 of 36 patients (8.3%) presented with acute rejection episode, no patient had steroid resistant rejection, and no grafts were lost. These results suggest that SRL-based immunosuppressive protocol is effective in preventing acute cellular rejection.

Renal dysfunction after OLT continues to plague the improvements in patients and graft survival among transplant recipients, and occurs in any different period after OLT. The incidence of chronic renal insufficiency in liver transplant recipients is 0.8% per year, ultimately leading to hemodialysis in 10% of cases and a mortality of 44%[20]. The nephrotoxicity following OLT is likely due to many factors, including hypotension during operation, infection, concomitant use of other nephrotoxic agents. However, the use of CNI such as tacrolimus is a major cause[21]. SRL appears to be devoid of nephrotoxic effects, while demonstrating comparable immunosuppression with CNI[22]. In group C, only after exclusion of other causes of nephrotoxicity and minimization of tacrolimus levels without graft rejection, could the patients be converted to SRL-based immunosuppression, suggesting that the improvement of renal function can not be derived simply from the tacrolimus dose reduction without graft rejection. Our results showed that serum creatinine level in nephrotoxicity group improved significantly after SRL therapy. All the 7 patients had complete normalization within 60 d after SRL treatment, suggesting that the observed improvement of renal function derives from both SRL conversion and tacrolimus dose reduction without graft rejection. Besides, in groups A and B, 29 patients who did not develop renal insufficiency during the SRL therapy further confirmed the non-nephrotoxicity of SRL.

The side-effect profile of SRL differs from that of CNIs. In our study, thrombocytopenia was easily reversible. Anemia could be controlled with blood cell transfusions, and recombinant erythropoietin. Oral aphthous ulcers were treated successfully with a reduced SRL dose and relief therapies. These adverse effects did not require discontinuation of SRL therapy and could be manageable. Hepatic artery thrombosis (HAT) is a dreaded complication, because it is often associated with allograft loss, patient death, or both. However, a single-center report with SRL and tacrolimus in University of Edmonton showed a low incidence of HAT (2%)[18]. In University of Colorado, Dunkelberg *et al*[23] noted that 170 patients with SRL treatment (5.3%) develop HAT compared with 8.3% of historical control patients. In both the Edmonton and the Colorado experience, the dose of SRL is modest (maintenance of 2 mg/d) and SRL levels are generally low (7 ng/mL). Considering that the adverse effects related to the administration of SRL are dependent on dose and concentration, we established the low target level of SRL in our group (5-8 ng/mL). With this approach, no episode of HAT was seen in our study. However, further investigation is necessary for elucidating the relationship between HAT and SRL.

In conclusion, SRL-based immunosuppression may improve CNI-induced renal insufficiency, inhibit the recurrence and metastasis of HCC, and prolong the patients' survival with good tolerability. However, further prospective studies with a large number of HCC patients with long-term SRL therapy are needed to confirm these findings.

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RAPID COMMUNICATION

Expression of trefoil factors 1 and 2 in precancerous condition and gastric cancer

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Abstract

AIM: To study the expression of trefoil factor 1 (TFF1) and TFF2 in precancerous condition and gastric cancer and to explore the relationship between TFFs and tumorigenesis, precancerous condition and gastric cancer.

METHODS: The expression of TFF1 and TFF2 was immunohistochemically analyzed in paraffin-embedded samples from 140 patients including 35 cases of chronic superficial gastritis (CSG), 35 cases of gastric ulcer (GU), 35 cases of chronic atrophic gastritis (CAG) and 35 cases of gastric cancer (GC).

RESULTS: TFF1 and TFF2 were located in cytoplasm of gastric mucous cells. In CSG, GU, CAG and GC, the level of TFF1 expression had a decreased tendency ($P < 0.05$). The expression of TFF2 was higher in GU than in CSG, but the difference was not significant. The expression of TFF2 also had a decreased tendency in GU, CAG, and GC ($P < 0.05$).

CONCLUSION: The reduced expression of TFF1 and TFF2 in precancerous conditions and gastric cancer may be associated with the proliferation and malignant transformation of gastric mucosa. More investigations are needed to explore the mechanism of TFFs and the relationship between TFFs and gastric cancer.

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Key words: Trefoil factor; Gastric cancer; Immunohistochemistry

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INTRODUCTION

Trefoil factor (TFF) is a newly discovered protective factor of gastrointestinal mucous, which has attracted more and more attention. This protein family consists of three members: TFF1 known as breast cancer-associated peptide (PS2), TFF2 as spasmolytic polypeptides (SP), and TFF3 as intestine trefoil factor (ITF). Human TFF1 and TFF2 contain only one trefoil structure domain, while human TFF3 contains two Trefoil structure domains[1]. pS2 (mpS2) gene is inactivated in the mouse, the antral and pyloric gastric mucosae of mpS2-null mice are dysfunctional and exhibit severe hyperplasia and dysplasia, while all homozygous mutant mice develop antropyloric adenoma, and 30% of them develop multifocal intraepithelial or intramucosal carcinomas[2]. Welter *et al*[3] found that TFF2 does not express in the pancreas but in pancreatic tumor of humans. Moreover, patients with pancreatic tumor expressing TFF2 have better prognosis. All these findings indicate that TFF is not only the repairing factor for inflammatory mucous and gastrointestinal ulcer, but also correlates with tumor development in digestive system. So far the functions and mechanism of TFF in gastrointestinal tract are unclear. The relationship between TFF and gastrointestinal tumors is still unclear. The aim of this study was to observe the features of the expression of TFF1 and TFF2 in four kinds of gastric mucosal lesion by immunohistochemistry and to explore the relationship between TFF1, TFF2 and tumorigenesis, progression of precancerous condition and gastric cancer.

MATERIALS AND METHODS

Materials

Thirty-five specimens of chronic superficial gastritis (CSG), chronic atrophic gastritis (CAG), gastric ulcer (GU) and gastric cancer (GC) were randomly taken from 150 patients in 1999-2000 (98 males and 42 females, the median age was 52.5 years). All the specimens were paraffin-embedded.

Immunohistochemistry staining

The streptavidin-peroxidase method was applied to immunohistochemistry. The anti-TFF1 protein antibody and the immunohistochemistry test kit (Kit-9710) were the products of Fuzhou Maixin Biotechnology Development

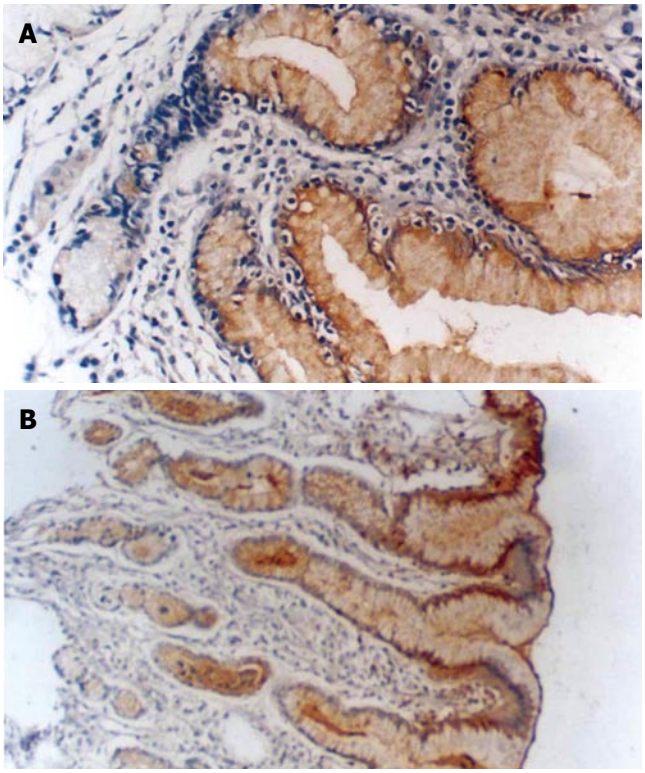


Figure 1 Strong yellow staining of TFF1 in superficial (**A**) and deeper (**B**) glands (SP100).

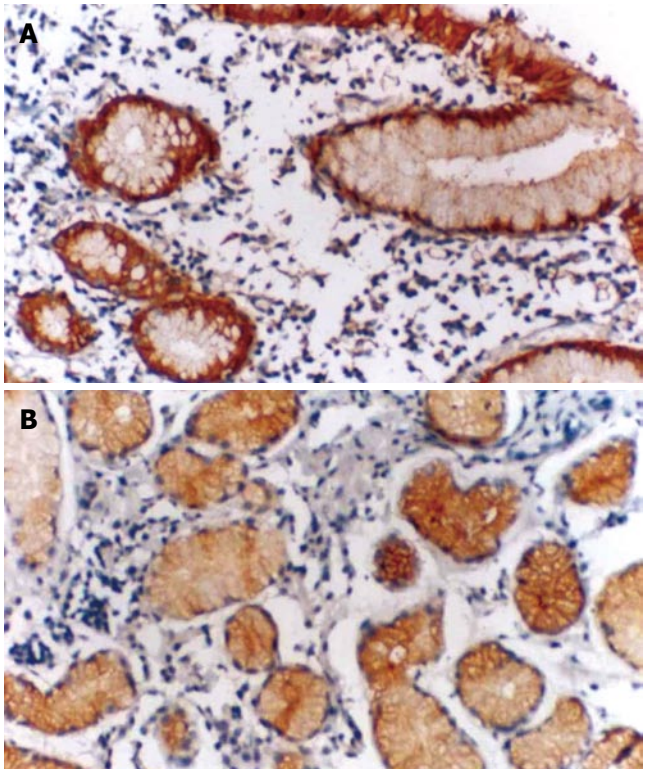


Figure 2 Strong brown-yellow staining of TFF2 in superficial (**A**) and deeper (**B**) glands (SP200).

Company. Anti-TFF2 protein antibody was provided by professor A.Giraud (Western Hospital, Australia). TBS (0.01 mol/L, pH7.4) was used as first antibody for contrast. The immunohistochemistry staining was performed according to the instructions of Fuzhou Maixin Biotechnology Development Company. DAB displayed the color. Yellow or brown-yellow pigmentation could be seen in cytoplasm and cell mucosa. According to the intensity of pigmentation and the range of positive reaction , we made the following scores: 0 = no staining or the staining intensity similar to the background; 1 = weakly positive or little deeper than the background, 2 = moderately positive or significantly deeper than the background, 3 = densely stained or deeply brown stained; According to the positive range, 0 = <10%, 1 = 10%-25%, 2 = 25%-50%, 3 = 50%-75%, 4 = >75%. Four classifications were made: 0-1 as (-), 2 as (+), 3-4 as (++) and above 5 as (+++).

Statistical analysis

All statistical analyses were carried out by SAS 8.0. Wilcox test was used according to the data. $P < 0.05$ was considered statistically significant.

RESULTS

Expression pattern of TFF1 in four kinds of gastric lesion

The expression of TFF1 could be detected in gastric mucosa. TFF1 was yellow or brown-yellow in positive regions and mainly located in cytoplasm. The expression of TFF1 could also be detected in most epithelia, glands and mucus around epithelial cell mucosa. Yellow staining in gastric foveola and superficial glands was relatively strong (Figure 1A). Yellow staining was stronger in superficial glands than in deeper ones and in basal gastric glands (Figure 1B). The level of TFF1 expression had a decreased tendency in different lesions of CSG, GU, CAG and GC ($P < 0.05$) and there was a significant difference between them ($P < 0.05$) (Table 1).

Expression pattern of TFF2 in four kinds of gastric lesion

Brown-yellow TFF2 was mainly located in cytoplasm. The expression of TFF2 could be detected in the mucus around epithelia, superficial and deep glands. However, the expression level was not significantly different between superficial and deep glands (Figure 2A). Yellow pigmentation was stronger in basal gastric glands (Figure 2B). The expression level of TFF2 in different gastric lesions was significantly different (Table 2). In different lesions, the expression level was GU > CSG > CAG > GC. There is no significant difference between CSG and GU. However, both of them were higher than CAG and GC ($P < 0.05$).

Relation between TFF1 and TFF2 expression and clinicopathological characteristics of GC

The level of TFF1 and TFF2 expression was lower in poorly-differentiated carcinoma than in moderately-and well-differentiated carcinoma ($P < 0.0001$, Table 3).

DISCUSSION

TFF is a small molecular protein with rich cysteine, and

Table 1 Expression of TFF1 protein by immunohistochmistry staining

Group	*n*	TFF1 expression				Mean score
		-	+	+ +	+ + +	
CSG	35	0	2	24	9	83.2
GU	35	1	6	20	8	73.7
CAG	35	2	3	27	3	68.1
GC	35	7	5	19	4	57

Table 2 Expression of TFF2 protein by immunohistochmistry staining

Group	*n*	TFF1 expression				Mean score
		-	+	+ +	+ + +	
CSG	35	0	0	22	13	80
GU	35	2	0	18	15	80.7
CAG	35	3	3	21	8	61.2
GC	35	2	5	21	7	60.2

Table 3 Expression of TFF1 and TFF2 in different GC

Type	*n*	TFF1 expression				Positive rate (%)	TFF2 expression				Positive rate(%)
		-	+	+ +	+ + +		-	+	+ +	+ + +	
Moderately-and well-differentiated carcinoma	21	0	2	15	4	81	0	0	14	7	100
poorly- differentiated carcinoma	14	7	3	4	0	50	2	5	7	0	85.7

is characterized by its sub-grade structures containing the trefoil like structure domain. This structure domain consists of 6 cysteine residues linked with 3 intramolecular disulfide chains. TFF is widely expressed in mucosa of endoblasts, especially in the gastrointestinal tract. In the normal gastrointestinal tract, TFF1 expresses mainly in gastric mucosa, TFF2 expresses mainly in gastric and intestinal mucosa, while TFF3 expresses mainly in intestinal mucosa. While the mucosa is acutely injured, this distinctive distribution disappears. TFF can protect against such injuries induced by stress[4], aspirin[5], indomethacin[6], frozen probe[7], *etc*. Alison *et al*[7] showed that when gastrointestinal mucosa is acutely injured, the expression of TFF increases around the injured mucosa. These findings suggest that TFF is a factor for acutely injured gastrointestinal mucosa. However, in this study, the level of TFF1 expression in chronic ulcer tissue was lower than that in chronic superficial gastritis ($P = 0.2587$), while the level of TFF2 expression in GU was slightly higher than that in CSG, but neither of which had any statistical significance, indicating that TFF can recover acute lesion of the mucosa but not chronic injury. The high expression of TFF1 and TFF2 in normal mucosa indicates that TFF1 and TFF2 are related with the physiological renewal of normal gastric mucosa cells.

TFF1 is first extracted from breast cancer cells. In breast cancer, the expression of TFF1 is estrogen-dependent. Recently, it was reported that the estrogen receptor is expressed in normal gastric membrane[8], gastric cancer[9] and pancreatic cancer[10,1]. However, Luqmani *et al*[12] found that the expression of TFF1 in normal gastric membrane and gastric cancer is non-estrogen dependent. The function of TFF1 in gastrointestinal tract and its relationship with estrogen are unclear. The decreased expression of TFF1 in gastric cancer and precancerous status indicates that TFF1 may be one of the inhibitory factors of gastric tumor and the decreased expression of TFF1 may be a sign of the deterioration of gastric membrane.

Human TFF2 contains two "trefoil" structure domains and does not express in breast cancer[13]. Pia Azarschab *et al*[14] found that aspirin (1-2mM) can significantly increase the expression of TFF2. However it is unclear whether this increasing can prevent gastric cancer. In this study, the expressive level of TFF2 in GU was higher than that in CSG, suggesting that TFF2 may play a more important role than TFF1 in the recovery of the gastric membrane. The expressive level of TFF2 in CAG and GC was lower than that in CSG ($P < 0.05$), suggesting that the expressive level of TFF2 decreases in gastric cancer and precancerous status. In this study, the common expression of TFF1 and TFF2 in mucous cells and epithelial mucous indicated that TFF interacts with mucous. TFF2 forms homodimers through cysteine in mucous gel layer[15,16], while TFF1 forms heterodimers in the same area[17,18]. Therefore, the viscosity of mucous is increased and the mucosa defensive barrier is stabilized. Because EGF, TFF1, and TFF2 are expressed in normal mucosa around the small intestinal ulcer, Wright *et al*[19] suggested that the expression of TFF1 is probably controlled by EGF. Yu *et al*[20] found that hydrotalcite increases expression of TFF2 mRNA, suggesting that hydrotalcite regulates TFF2 mRNA expression through the increase of epidermal growth factor

The expressive level of TFF1 and TFF2 was higher in gastric cancer but lower in poorly-differentiated carcinoma than in well-differentiated carcinoma, indicating that decreased expression of TFF1 and TFF2 not only has a close relationship with the occurrence of gastric cancer, but also relates to the malignancy of gastric cancer. So far the specific mechanism of these two proteins in normal gastric mucosa and in the process of malignancy is unclear. Decreased expression of TFF1 and TFF2 may be associated with the proliferation and malignant transformation of gastric mucosa. The results of this study may bring some enlightenments to the prophylaxis and treatment of gastric cancer.

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RAPID COMMUNICATION

Primary shunt hyperbilirubinaemia in a large four-generation family confirming autosomal dominant genetic disorder

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Abstract

AIM: To describe the pattern of inheritance and confirm the diagnostic criteria of primary shunt hyperbilirubinaemia (PSH).

METHODS: Forty members of a family pedigree across four generations were included in this study. All family members were interviewed and investigated by physical examination, hematology and liver function test and the pattern of inheritance was analyzed.

RESULTS: Nine of the forty family members suffered primary shunt hyperbilirubinaemia. The mature erythrocytes of the propositus were irregular in shape and size. The pedigree showed transmission of the trait through four generations with equal distribution in male and female. No individual with a primary shunt hyperbilirubinaemia was born to unaffected parents. The penetrance was complete in adult.

CONCLUSION: The pattern of inheritance is autosomal dominant. The abnormality of erythrocytes and decrease in white blood cell could be supplemented in the diagnosis of PSH. The PSH is a genetic disorder and could by renamed as hereditary shunt hyperbilirubinaemia.

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Key words: Hyperbilirubinaemia; Hereditary; Shunt

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INTRODUCTION

Primary shunt hyperbilirubinaemia (PSH) is a rare clinical jaundice characterized by an elevated unconjugated serum bilirubin, hyperplastic/ineffective erythropoiesis with a normal peripheral red blood cell survival[1]. From the first case described by Israels in 1959, less than 30 PSH cases have been reported in world literature. Most of them were sporadic reports with only 2 small familial analysis[2,3,4]. We present herein a large family pedigree in which the propositus characterized some different from the previous reports and the inheritance pattern was determined to be autosomal dominant, and in which PSH could probably be renamed as hereditary shunt hyperbilirubinaemia.

MATERIALS AND METHODS

Case report

The propositus is a 28-year-old Chinese man who was admitted to our hospital because of asymptomatic fluctuating jaundice observed over the previous five years. At age 23, the patient first presented with elevated serum unconjugated bilirubin, splenomegaly and anemia. The serum bilirubin fluctuated between 50-90 μmol/L. There was no history of infectious hepatitis, malaria, Schistosomiasis or other significant childhood illness. He denied any alcohol intake and was taking no medications at the time of admission to our hospital and worked in a normal fashion as a farmer.

Physical examination revealed a well-developed man of normal stature and growth. Moderate skin icterus was present with mild pallor sclera. The spleen was palpable 4.0 cm below the left costal margin. The liver was impalpable and of normal size on ultrasonography. Murphy's sign was negative. There was no ascites or peripheral edema, and the neurologic examination was normal (Figure 1A and B).

Initial hematology showed the hemoglobin to be 78 g/L, red blood cell count 3.67×10^{12}/L, MCV 97.5 fl, MCH 34.0 pg, white blood cell count 2.7×10^{9}/L and platelets 226×10^{9}/L. The reticulocyte count was 3.0%. The differential white cell count was normal. The peripheral smear showed irregular erythrocytes in shape and size with mild macrocytosis and target-, rod-, tear-drop cells (Figure 1C). Total serum protein and albumin were 71.0 g/L and 46.7 g/L, respectively. Alanine aminotransferase (ALT) and serum lactic dehydrogenase (LDH) were normal. Total and conjugated serum bilirubin were 74.2 and 8.8 μmol/L, respectively. Alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGT) were normal. Urine urobilinogen

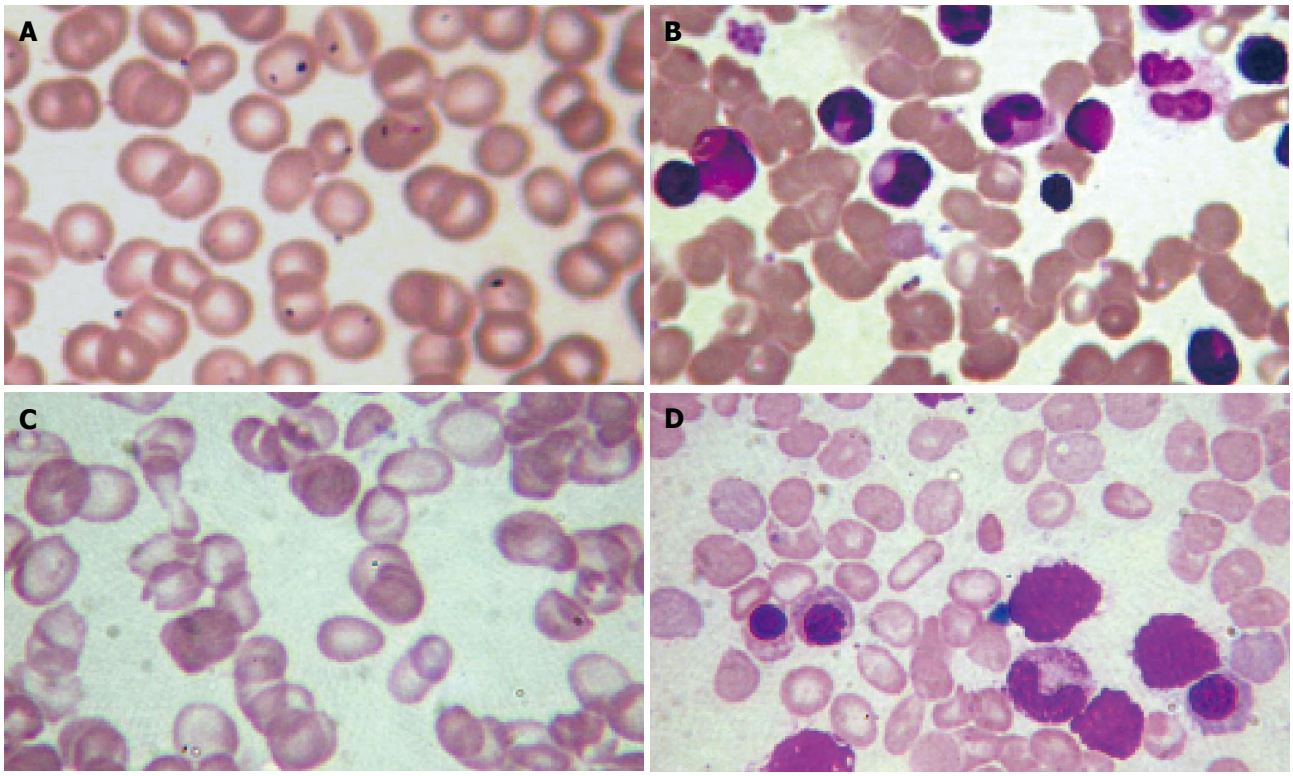


Figure 1 Peripheral smear and bone marrow aspirate. **A** and **B**: Peripheral smear and bone marrow aspirate of a healthy volunteer, respectively (400×, Giemsa); **C**: Peripheral smear of the propositus, showing irregular erythrocytes in shape and size with mild macrocytosis and target-, rod-, tear-drop cells; **D**: Bone marrow aspirate of the propositus which revealed a erythroid hyperplasia and some target-, rod-, tear-drop erythrocytes.

was positive. The serum haptoglobin was 0.06 g/L. Serum iron and total iron binding capacity were normal. Glucose-6-phosphate dehydrogenase, pyruvated kinase was normal, as were the Ham's test and Coombs' test. The hemoglobin electrophoresis, serum protein electrophoresis, autohemolysis, osmotic fragility (before and after incubation) were all normal. The urine for hemosiderin was negative. Administration of phenobarbital (60 mg/24 h) for one week did not reduce plasma bilirubin to the baseline level (total bilirubin 68.3-64.6 μmol/L).

The bone marrow aspirate revealed a hypercellular marrow due to an erythroid hyperplasia. Of particular interest is in which there were some target-, rod-, tear-drop cells erythrocytes consistent with the peripheral smear (Figure 1D).

Pedigree analysis

The symptoms and signs of the all members through four generations were thoroughly inspected by two gastroenterologists respectively. Four to six milliliter blood samples of each member were collected using potassium citrate as coagulating agent. The peripheral blood cell count and ALT, total serum bilirubin, serum unconjugated bilirubin, serum albumin, blood urea nitrogen, creatinine were detected.

RESULTS

In our patient's pedigree, the grandfather, mother and maternal aunt showed recessive jaundice (Figure 2). Nine of them suffered from splenomegaly and had elevated unconjugated bilirubin. Among these suffered persons, 5 showed decreased white blood cell counts and 4 showed decreased hemoglobin simultaneously. The patient's grandfather (Ⅰ-1), mother (Ⅱ-2), maternal aunt (Ⅱ-4), three elder brothers (Ⅲ-5, Ⅲ-7, Ⅲ-9), one cousin (Ⅲ-14), two nieces (Ⅳ-7, Ⅳ-15) had clinical features practically identical to that of the propositus. The patient's immediate family had definitive laboratory corroboration and the same clinical entity distributed equally in male and female.

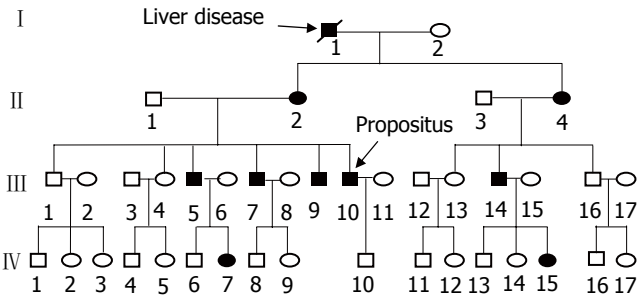


Figure 2 The four-generation family pedigree affected by PSH. The propositus is indicated with an arrow. Symbols are indicated as follows: Circles for females; squares for males; blank for unaffected; full shadowed for affected by PSH.

DISCUSSION

Shunt hyperbilirubinaemia is a subtype of unconjugated hyperbilirubinaemia and it describes the increase in bilirubin production associated with ineffective erythropoiesis. It has been proposed that ineffective erythropoiesis is related to progenitor inhibition, abnormality on erythro-

poietin (Epo)/Epo receptor signaling, *etc*. Secondary shunt hyperbilirubinaemia due to ineffective erythropoiesis can be seen in a number of common clinical disorders, such as thalassemia, myelodysplastic syndrome (MDS) and congenital refractory anemia[5-8]. PSH is a rare disorder with laboratory findings of elevated unconjugated serum bilirubin, mild reticulocytosis, erythroid hyperplasia of bone marrow, splenomegaly and normal peripheral red blood cell survival in the young people[1]. Although some sporadic cases were reported since 1959, the molecular genetic basis has not been previously described because of little pedigree information.

In our case, the clinical findings of the propositus were consistent with the diagnosis of PSH. It is of extreme interest that our patient is unusual in that some abnormal erythrocytes appeared in peripheral smear and bone marrow aspirate, whereas most other reported cases were characterized by normal erythrocytes. It can be proposed that PSH is related to abnormal development of erythrocyte and intramedullary ineffective erythropoiesis. Moreover, the finding of decreased white cell count with normal reticulocyte count indicated the coexistence of splenomegaly and hypersplenism. The abnormal erythrocytes and hypersplenism can be presented as additional clinical findings in PSH diagnosis.

The family pedigree we reported covered four generations. In our patient's pedigree, the grandfather, mother and maternal aunt showed recessive jaundice. Five of eight (60%) adults in the third generation suffered from this defect. The patient's immediate family had definitive laboratory corroboration and it appears that they were suffering from the same clinical entity which distributed equally in male and female. It is speculated that the transmission of defect in our patient's pedigree is an autosomal dominant with equal distribution in male and female. No individual with a PSH was born to unaffected parents. That is, the penetrance was complete in adult. But the severity of clinical features of the disorder was not the same among these affected individuals. Although Bird *et al*[4] reported that the pattern of inheritance of HSP was X-linked transmission[4], the family pedigree was too small to determine the pattern of inheritance. Here we have provided the first evidence that PSH can be inherited as an autosomal dominant Mendelian trait at least in some cases. Nevertheless, we failed to show male to male transmission of definitely affected individuals which would increase the possibility of chance association. Probably it is associated with the incomplete penetrance in the 4th generation. It remains to be determined whether that refers to all cases of PSH or whether there is a spectrum of different diseases.

Up to now, the precise defect of PSH at the cellular and molecular level remains obscure, although the pathogenesis of PSH centers on markedly ineffective erythropoiesis. Our patient's pedigree consists of 4 generations, 29 members, and 9 patients. As the largest pedigree of PSH, this family would be suitable for genome-wide scanning to allow linkage or mapping of suitable PSH genes.

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LETTERS TO THE EDITOR

This year's Nobel Prize to gastroenterology: Robin Warren and Barry Marshall awarded for their discovery of *Helicobacter pylori* as pathogen in the gastrointestinal tract

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TO THE EDITOR

Peptic ulcer disease is a major health care concern in the society today, in view of personal suffering as well as economical health care costs.

Before *Helicobacter pylori* (*H pylori*)

During the last century peptic ulcer research was much focused on the understanding of the relationship of peptic ulcers to acid secretion. The dictum of Karl Schwarz: "No acid - no ulcer" provided the framework for ulcer research.

Surgery aiming at reducing gastric acid secretion is a common treatment of choice, as drugs such as anticholinergics and antacids are not good enough in achieving a reliable treatment response. The surgery involves selective and proximal vagotomy of the parietal cell mass, removal of the gastrin-producing antrum, or combinations of the two. The most extensive operations carried out are the Billroth I and Billroth II operations which regularly remove the antrum, alone or in combination with vagotomy and gastroenteroanastomosis. Both operations involve major surgery and often postoperative complications.

Since the invention of the histamine H_2-receptor antagonists in 1974, the peptic ulcer research field has been even more emphasized and the drug industry has converged on its power on the inhibition of gastric acid secretion as a cure for peptic ulcer disease. This was further encouraged by the 1988 Nobel Prize in physiology or medicine for important principles in drug treatment. The principles that were awarded have resulted in the development of drugs with antagonist action, the acid-inhibitory H_2-receptor antagonist cimetidine is among them.

Along this line even more efficient acid inhibitors that block the H^+,K^+-ATPase, the proton pump, have been developed. To mention one, omeprazole was the first to surface on the arena at the World Congresses of Gastroenterology in 1982, yet some years before taken to the clinic. This compound was later shown to be able to bring down acid secretion to nil and heal ulcers more rapidly than H_2-receptor antagonists. To conclude, the "no acid, no ulcer" concept is consolidated among gastroenterologists as the problem of peptic ulcer appears to be resolved with chronic administration of antisecretory drugs. However, when treatment is withdrawn the ulcers recur. Since then, the recurrence has been considered an effect of receptor up-regulation after long-standing H_2-receptor blockade or persistent hypergastrinemia after treatment with a proton pump inhibitor[1].

Infectious etiology of peptic ulcer disease

An association between gastritis and peptic ulcer disease has been known since the 1940s. Efforts to culture these bacteria however have failed. The Australian histopathologist Robin Warren, in Perth, being unaware of the earlier findings has made similar observations but also the observation that the appearance of these bacteria is similar to the *Campylobacter* genus and preliminary named the bacterium *Campylobacter pylori*[2].

Barry Marshall, being a trainee in internal medicine working in Stewart Goodwin´s Laboratory in Perth, Australia, was with serendipity successful in culturing the bacteria. Over the Easter holiday the Petri dishes with bacterial cultures were inadvertently left in the incubator in five, instead of two days, as recommended for *Campylobacter*. When the dishes were examined after the holidays, small colonies with a shiny appearance were detected. This is the first successful culture and isolation of a bacterium from the stomach of a patient with gastritis. The findings were presented at a Campylobacter meeting 1983[3]. Initially the reports of an association between the bacterium and gastritis were met with great scepticism from the established scientific community. The findings were later published in the Lancet[3].

One major question is whether the bacterium is the cause of gastritis or simply an innocent bystander that happens to colonise a damaged mucosa. In order to rule this out, Barry Marshall and Arthur Morris decided to inoculate themselves by drinking a solution containing vast amounts (10^9) of the bacterium under acid suppression with cimetidine. Both developed signs of an acute gastric "flu"-like illness, with gastric distension, nausea and vomiting. After 10 days endoscopy revealed contraction of *H pylori* and chronic gastritis[4], which gradually subsided over the next

two weeks. Renewed endoscopy could not disclose the organism and the gastritis was healing. In the case of Morris the symptoms remained as a common gastritis. These cases are the ones to confirm the Koch´s postulate that the bacterium itself indeed is the cause of disease[5].

Clinical trials in order to eliminate the bacterium from the stomach showed that treatment with antibiotics could reduce the symptoms and severity of gastritis. Studies also showed that eradication of *Helicobacter* could prevent the relapse of peptic ulcer disease[6]. This has generated a continuing flow of research from the scientific community, which emanated in the acceptance of gastritis and peptic ulcers as basically infectious diseases. The cause of ulcer disease has previously been hidden by a dual false premise that the stomach is sterile and ulcers are caused by stress. This dogma is so deep in scientific thinking that it has taken more than a decade of research and repeat replication of results by scientists to have this fact accepted.

H pylori causes life-long infection

H pylori is a spiral-shaped Gram-negative bacterium that colonizes the stomach. In countries with high socioeconomic standards infection is considerably less common than in developing countries where virtually everyone may be infected.

Infection is typically contracted in early childhood, frequently by transmission from mother to child, and the bacteria may remain in the stomach for the rest of the person's life. This chronic infection is initiated in the antrum of the stomach. As first reported by Robin Warren, the presence of *H pylori* is always associated with an inflammation of the underlying gastric mucosa as evidenced by an infiltration of inflammatory cells[2].

The infection is usually asymptomatic but can cause peptic ulcer

The severity of this inflammation and its location in the stomach are of crucial importance for diseases that can result from *H pylori* infection. In most individuals *H pylori* infection is asymptomatic. However, about 15% of infected individuals will some time develop peptic ulcer disease. The current view is that the chronic inflammation in the distal part of the stomach caused by *H pylori* infection results in an increased acid production from the non-infected upper corpus region of the stomach. This will predispose for ulcer development in the more vulnerable duodenum. After the discovery of *Helicobacter* as the pathogen, a 7-day course of antibiotics in conjunction with acid suppression may heal a previously chronic disabling disease.

Malignancies associated with H pylori infection

H pylori can also infect the corpus of the stomach. This results in a more widespread inflammation that predisposes not only to gastric ulcers in the corpus, but also to stomach cancer. Globally, this cancer has decreased in incidence in many countries during the last 50 years but is still ranked as number two in the world in terms of cancer deaths after lung cancer. Mucosa associated lymphoid tissue (MALT) lymphoma is a specific type of lymphatic neoplasm in the stomach that has been associated with *H pylori*. Curiously, MALT lymphomas regress when *H pylori* is eradicated by antibiotics, why the bacterium seems to play an important role in the development of this tumour.

Microbial origin of chronic inflammatory conditions in general?

The discovery that one of the most common diseases of mankind, peptic ulcer disease has a microbial cause, has stimulated the search for microbes as possible causes of other chronic inflammatory conditions. Even though no definite answers are at hand, recent data clearly suggest that a dysfunction in the recognition of microbial products by the human immune system can result in disease development. The discovery of *H pylori* opens the possibility of an increased understanding of the connection between chronic infection, inflammation and cancer.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
Berlin
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symposia@falkfoundation.de

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6–7 May 2006
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ILTS 12th Annual International Congress
3–6 May 2006
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Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
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6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
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www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
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c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
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European Society of Clinical Microbiology and Infectious Diseases
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26–29 April 2006
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www.ddw.org

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25–26 May 2006
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American Society of Gastrointestinal Endoscopy
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3 **Lin GZ,** Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 285-287

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1	4 days	4 d	In figures, tables and numerical narration
2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
5	2 hours	2 h	After Arabic numerals
6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
8	10 seconds	10 s	After Arabic numerals
9	10 year	10 years	In text narration
10	Ten yr	Ten years	At the beginning of a sentence
11	0,1,2 years	0, 1, 2 yr	In figures and tables
12	0,1,2 year	0, 1, 2 yr	In figures and tables
13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
15	2 months	2 mo	In figures and tables
16	Two mo	Two months	At the beginning of a sentence
17	10 minutes	10 min	
18	Ten min	Ten minutes	At the beginning of a sentence
19	50% (*V/V*)	500 mL/L	
20	50% (m/*V*)	500 g/L	
21	1 M	1 mol/L	
22	10 μM	10 μmol/L	
23	1N HCl	1 mol/L HCl	
24	1N H_2SO_4	0.5 mol/L H_2SO_4	
25	4rd edition	4^{th} edition	
26	15 year experience	15- year experience	
27	18.5 kD	18.5 kDa or *Mr*18 500	
28	25 $g.kg^{-1}/d^{-1}$	25 g/kg per day	
29	96000	96 000	
30	1000 rpm	1000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	1 pg/L	
33	10 kilograms	10 kg	
34	Gene bank	GenBank	International classified genetic materials collection bank
35	Ten L	Ten liters	At the beginning of a sentence
36	Ten mL	Ten milliliters	At the beginning of a sentence
37	umol	μmol	
38	30 sec	30 s	
39	1 g/dl	10 g/L	10-fold conversion
40	OD_{260}	A_{260}	"OD" has been abandoned
41	One g/L	One microgram per liter	At the beginning of a sentence
42	*A*260 nm	A_{260} nm	*A* should be in italic
43	^bP<0.05	^aP < 0.05	In Table, no note is needed if there is no significance instatistics: ^aP<0.05, ^bP<0.01 (no note if P>0.05). If there is a second set of P value in the same table, ^cP<0.05 and ^dP<0.01 are used for a third set: ^eP<0.05, ^fP<0.01.
44	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F = 9.87, ^2F = 25.9 ^3F = 67.4	Notices in or under a table
45	KM	km	kilometer
46	CM	cm	centimeter
47	MM	mm	millimeter
48	Kg, KG	kg	kilogram
49	Gm, gr	g	gram
50	nt	N	newton
51	l	L	liter
52	db	dB	decibel
53	rpm	r/min	rotation per minute
54	bq	Bq	becquerel, a unit symbol
55	amp	A	ampere
56	coul	C	coulomb
57	HZ	Hz	
58	w	W	watt
59	kPa	mmHg	
60	p	Pa	pascal
61	ev	EV	volt (electronic unit)
62	Jonle	J	joule
63	J/mmol	kJ/mol	kilojoule per mole
64	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
65	N·km	KN·m	moment
66	$x\pm s$	mean ± SD	In figures, tables or text narration
67	Mean±SEM	mean ± SE	In figures, tables or text narration
68	*im*	im	intramuscular injection
69	*iv*	iv	intravenous injection
70	Wang et al	Wang *et al*	
71	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing
72	Ecoli	*E coli*	Bacteria and other biologic terms have their specific expression
73	Hp, *Helicobacter pylori*	*H pylori*	
74	Iga	*Iga*	writing form of genes
75	*igA*	IgA	writing form of proteins
76	5 mg/kg/h	5 mg/kg per hour	
77	0-29%	0%-29%	
78	Number, No, No. cases, number of mice, number of cases	*n*	In figures and tables

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READING GUIDE

In the following 20 articles we have attempted to give an update and overview of different aspects of gastrointestinal disease that are treated via a surgical intervention. In addition we have included topics related to advances and innovations in surgical instrumentation and training that are useful in treating gastrointestinal disease. Additional topics covered deal with the ethics of abdominal organ transplantation, inflammatory bowel disease in adolescents and children, and discussions of the quality of life following gastrointestinal cancer. We have also included a discussion of recent discoveries involving the calcium sensing receptor and how this receptor may be employed as both an alternative therapy as well as a possible preventative measure for a variety of gastrointestinal disease issues. As we hope becomes apparent from this collection of articles that the surgeon has and will remain an integral part of the identification and treatment of gastrointestinal disease. We have also included a topic on the surgical scientist and how the surgeon of the 21st century has become an active player in the bench side investigations of causality for gastrointestinal disease.

It is our hope that this collection of articles will give the reader an in depth understanding of the role of the surgeon in all aspects of patient management; from identification of disease related factors, to the removal or repair of the effected tissue, to the postoperative counseling and management of the patient.

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EDITORIAL

Management of parastomal ulcers

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Abstract

Management of surgically placed ostomies is an important aspect of any general surgical or colon and rectal surgery practice. Complications with surgically placed ostomies are common and their causes are multifactorial. Parastomal ulceration, although rare, is a particularly difficult management problem. We conducted a literature search using MD Consult, Science Direct, OVID, Medline, and Cochrane Databases to review the causes and management options of parastomal ulceration. Both the etiology and treatments are varied. Different physicians and ostomy specialists have used a large array of methods to manage parastomal ulcers; these including local wound care; steroid creams; systemic steroids; and, when conservative measures fail, surgery. Most patients with parastomal ulcers who do not have associated IBD or peristomal pyoderma gangrenosum (PPG) often respond quickly to local wound care and conservative management. Patients with PPG, IBD, or other systemic causes of their ulceration need both systemic and local care and are more likely to need long term treatment and possibly surgical revision of the ostomy. The treatment is complicated, but improved with the help of ostomy specialists.

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Key words: Parastomal ulcers; Peristomal ulcers; Ostomies; Complications

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INTRODUCTION

Creating and managing enteral stomas is an integral part of many surgical practices. Ostomy outcomes depend on initial optimization of placement and wound care and the circumstances under which the stoma is created (emergent *vs* elective). Unfortunately, even with appropriate technique in the placement of an ostomy, complications are fairly common. Parastomal ulceration, although a rare complication, can prove particularly troublesome. A review of the literature shows many management options but little in the way of definitive care.

An ostomy is a surgically placed opening in the bowel that is brought to the surface of the abdominal wall. The term stoma refers to the actual end of the portion of bowel that comes through to the surface of the abdominal wall, based on the Greek word for mouth or opening. Ostomies themselves may be temporary or permanent, loop or end, and made from small bowel or colon. They can be used as definitive treatment or as a temporary diversion to permit inflammation to resolve or allow an anastomosis to heal. They are used for the management of a variety of pathological conditions, including congenital malformations, cancer, trauma, inflammatory conditions, and ulcerative colitis. They may be exteriorized on the neck, chest, or abdomen. The abdomen is the most common site utilized. Esophagostomy, gastrostomy, jejunostomy, and cecostomy are usually only used temporarily. Colostomy, ileostomy, and ileal conduits are used for more extended periods of time. While each type of ostomy has different characteristics and maintenance needs, they all have complications, many of which can be managed in a similar manner.

TYPES OF OSTOMIES

Colostomy

A colostomy is performed when it is necessary to bypass or remove the distal colon and or rectum. A sigmoid colostomy may be used to divert the fecal stream for anorectal disease or after proctectomy in cases in which the colon is spared of disease. Other indications for either a permanent or temporary colostomy include diverticulitis, congenital anomalies, colorectal trauma, and Crohn's disease.

The most common indication for performing a permanent sigmoid colostomy or end colostomy is carcinoma of the rectum. If the distal rectum and anorectal sphincter mechanism are removed, the colostomy is permanent. Endoanal stapling devices have reduced incidence of this type of ostomy because they permit a low anastomosis in a setting where a hand-sewn anastomosis would not have been possible.

A proximal or transverse colostomy is often used as a temporary basis for obstructing or perforating lesions of the colon, trauma, anastomotic leak, segmental colonic resection to protect an anastomosis when residual disease or sepsis is present, or more rarely for congenital anomalies. Decompressing colostomies are constructed by bringing the loop of bowel to the abdominal surface and creating an opening through the anterior abdominal wall. This provides a mechanism for evacuating fecal contents and also permits access to the obstructed bowel for subsequent resection.

A diverting colostomy may also be performed in order to divert the fecal stream proximal from an area of pathology. In this setting, a fecal diversion promotes healing of the involved site. There are several approaches to the construction of a temporary diverting colostomy. Both ends of the transected bowel can be brought to the abdominal wall as stomas; the proximal end can be brought to the abdominal wall as an end colostomy and the distal end may be over-sewn and left the abdomen (Hartmann's procedure). Alternatively, a primary anastomosis can be performed and a proximal loop can be brought to the abdominal wall for protection of the anastomosis.

Cecostomy

Historically, cecostomy has been advocated to protect a left sided anastomosis, used as a treatment for large bowel obstruction, cecal perforation, volvulus or Ogilvie's syndrome. However, its use nowadays is extremely limited[1]. Cecal volvulus must be treated surgically and is usually treated with ileocolonic resection with ileostomy and mucus fistula. However, if the patient's condition is such that resection is not advisable due to comorbidities, such as if the patient is in frail condition, immunosuppressed, or critically ill, then the best option is to place a cecostomy tube. The purposes of a cecostomy are two-fold. First, it effectively decompresses the cecum, whose distention was a contributing factor in the development of the volvulus. Second, it anchors the cecum to the anterior abdominal wall, which prevents recurrence of the volvulus. This second effect is also known as "pexing" of the cecum[2]. A cecostomy is a temporizing decompressive procedure and does not divert the fecal stream.

Enterostomy

Ileostomy is most often performed after total proctocolectomy for Crohn's disease, familial polyposis, or other conditions requiring the removal or bypass of the entire colon and rectum. If the anorectal sphincter is removed, the ostomy will be permanent, if it is retained it has the potential for reversal. A standard ileostomy may be constructed as either a loop or end stoma. A continent ileostomy is constructed by detubularizing a segment of terminal ileum and reconfiguring it as a reservoir.

An end ileostomy, or Brooke Ileostomy, is the standard of care for patients requiring pan-proctocolectomy. The Brooke technique involves the eversion of bowel to expose the mucosa followed by mucocutaneous suturing to create an end stoma in order to prevent the development of serositis and stricture formation.

A loop ileostomy used to provide temporary fecal diversion following sphincter-saving procedures. Loop ileostomies are typically more difficult to manage because the stoma more frequently empties close to the skin. In addition, a loop ileostomy may be performed more proximally in the ileum and may be associated with higher enzyme and fluid output.

Continent ileostomies are formed after colectomy. A reservoir pouch and valve are created using ileum and a stoma is brought up to the skin; these allow for a storage place for stool and some control of continence. After surgery, patients empty the pouch by using a tube through the ostomy site. This procedure is often used in ulcerative colitis where it is a curative procedure.

OSTOMY PLANNING AND CREATION

Preoperative planning is important in determining ostomy outcomes. Optimal site selection is an important part of stoma construction. Poor stoma placement may cause difficulties with appliance fit. This, in turn, may cause leakage leading to clinically important skin irritation or breakdown around the ostomy[3]. Marking the site must take into account the patient's body habitus and physical limitations as well as any supports or braces utilized by the patient. The patient must be able to visualize and reach the stoma. Skin folds should be avoided.

The location of the stoma is the most important factor contributing to subsequent morbidity and requires marking the site prior to surgery to ensure optimal placement. Marking may be performed by surgeons or trained enterostomal therapists. Proper location of the stoma can reduce or prevent complications.

Enterostomies can be brought through the abdominal wall in laparotomy incisions or through a separate site. Some of the disadvantages of bringing a stoma through a large laparotomy incision include the risk of wound infection, dehiscence, and evisceration. Some stomas are created through the incision, especially when the only goal of surgery is to create a stoma (e.g. PEG placement).

Generally, the stoma should be placed within the triangle formed by lines between the umbilicus and the anterior superior iliac spine, the umbilicus to the pubis, and anterior superior iliac spine to pubis (inguinal ligament), but exceptions exist. For example, the waistline may pose a problem. Typically, in obese patients the stoma should be sited at a higher level, which permits these patients to directly visualize and to care for the stoma optimally. Usually the stoma is placed at the summit of the infraumbilical bulge and within the rectus muscle. The groin, costal margins, waistline, and scars should be avoided because they commonly interfere with appliance management. Consequently, patients should be assessed when standing, sitting, and lying to ensure that the appliance sits properly and the risk for leakage is avoided. In obese patients the stoma should be situated at a higher level of the abdomen so that the patient is able to visualize and access the stoma.

The opposite side of the abdomen should always be

marked, especially in individuals with IBD. As the final amount and area of bowel resection at the time of surgery may be different from originally planned and not allow for creation of the ostomy on the originally planned site. If the terminal ileum has been resected, it is usually easier to bring the stoma out in the left lower quadrant rather than the right. Other locations may be used if appropriate. For example, a jejunostomy may necessitate placement in the upper abdomen.

COMPLICATIONS

Ostomies have a high risk of complications, which are not only related to stoma location or type[4]. Obesity and IBD predispose to complications.

Complications include intestinal obstruction, stenosis, retraction, prolapse, skin irritation, diarrhea, kidney stones, gallstones, ileitis, varices, parastomal abscess or fistula, and parastomal ulceration[5]. Frequency of ostomy complications are in the range of 6%-26%[6,7].

Peristomal skin breakdown/irritation

(Mechanical trauma, effluent contact damage, allergic reactions, fungal infections) Skin irritation is a common problem in patients with stomas. It is more common in ileostomies than colostomies and is seen in 30%-40% of patients[4]. It may be secondary to contact dermatitis, mechanical irritation, trauma, infection, or peristomal hyperplasia. The mainstay of treatment is local wound care and identifying the underlying cause of the problem. Immunosuppressed and diabetic patients are more susceptible to fungal infections.

Parastomal herniation

Parastomal herniation occurs in approximately 10%-30%[8] of all stomas and occurs more often in colostomies than ileostomies. Most of these should be repaired if symptomatic, but because of the incidence of local recurrence, they present a difficult problem. Surgical options include local repair with mesh or relocation. Actual etiological factors include poor wound healing, poor abdominal muscle tone, poor placement of the stoma, poor surgical technique, including too large a fascial opening.

Stomal stenosis

Stomal stenosis usually occurs secondarily to ischemia, infection, or a retracted stoma. The incidence is approximately 10%. Management may require either dilation or revision of the stoma. Dilation itself leads to additional scarring and is only a temporary measure. Dietary modifications may alleviate complications from stomal stenosis.

Stomal retraction

Retraction of a stoma leads to difficulty with appliance placement and as a result leads to skin irritation adjacent to the stoma. The use of a convex appliance often helps outcome and may prevent leakage and skin breakdown.

Stomal prolapse

Stomal prolapse can occur in both end and loop stomas. Both the proximal and distal bowel segments can protrude, although prolapse appears to be more common in the distal segment[9]. The rate of stomal prolapse is 7%-11%[9]. Improvement in surgical techniques and operative placement have reduced the incidence of prolapse. Sizing of the stoma and placement within the rectus muscle are important. Nutritional status of the patient may also affect outcome. In most cases the prolapse is just a cosmetic problem, but may result in strangulation or obstruction if large amounts of bowel herniate. Reduction of an incarcerated stoma may be achieved by the use of an osmotic agent such as sugar or salt, which helps reduce edema. Ice may also be used to reduce swelling. In rare cases, however, surgical revision is indicated for ischemia, obstruction, ulceration or chronic bleeding.

Obstruction

Stomal stricture can occur at the skin and/or fascial level. A stricture may be the result of scarring, inflammation, or technical error. Obstruction can lead to hyperperistalsis and hypersecretion, and subsequent fluid losses resulting in massive dehydration. Most severe stoma strictures require surgical revision.

Peristomal varices/bleeding

Peristomal varices can be seen in patients with stomas and any cause of portal hypertension. Acute management includes direct pressure and injection of sclerosing agents. Recurrence is frequent and usually involves portalcaval shunting in patients who are surgical candidates.

PREVENTION AND OSTOMY CARE

Hughes *et al* found that optimizing the perioperative health status of the patients, particularly the elderly, may reduce morbidity and complication rates. Their results also support specialist surgical care of patients undergoing stomal surgery[5]. Enterostomal nursing can be instrumental in preventing complications and in decreasing the frequency of peristomal complications. Proper fitting of appliances, leakage control, and early local wound care lead to lower morbidity and better quality of life[4].

PERISTOMAL ULCERATION

Symptoms and etiology

Chronic parastomal ulcers in patients with ileostomies or colostomies are unusual and difficult to manage. Many causes have been attributed to parastomal ulceration including infections, fistulas, recurrent inflammatory bowel disease, pyoderma gangrenosum and trauma. Parastomal ulcers occur most commonly in patients with Crohn's disease but have also been observed in patients with mucosal ulcerative colitis. The main symptoms are pain and difficulty with appliance placement. Patients often complain of pain that is quite severe, and may be out of proportion to physical findings. It is usually described as

burning. On examination, erythema and induration with a pink halo are seen. There is often drainage from the mucocutaneous junction, but no overt collection.

Parastomal ulcers that occur within the first few months after stoma construction are more likely to be attributable to operative technique, but may also be due to low grade infection at the site. Those that evolve later are less likely to be associated with the stoma site itself and are more likely caused by recurrence of IBD or chronic infection that only becomes apparent with a weakened host system[10].

Ramanujam *et al* report an unusual case of a squamous cell carcinoma arising at the ileocutaneous stomal site[11]. The patient presented with peristomal ulceration and bleeding and was treated with a wide local excision of the stoma and relocation of the ileostomy. Only two other cases of this have been reported in the literature[11]. Therefore, although rare, one must always consider a malignancy if a peristomal ulcer does not heal after aggressive medical management.

MEDICAL MANAGEMENT

Management of ulceration at the stomacutaneous junction is mostly medical consisting of local wound care, proper appliances, and local and systemic corticosteroids[10]. Wolfsen and colleagues reviewed ten cases over eight years of refractory parastomal ulcers. Eight of the ten patients had ileostomy placement for IBD while the other two had undergone colostomy for colon cancer. Five of the patients were diagnosed as having peristomal pyoderma gangrenosum. Those five patients required a mean of 25 wk of systemic steroid or IBD therapy before resolution of their parastomal ulcers. The other patients had ulcers that were due to contact ulcers from appliances, or dermatoses. These patients received traditional stomal care and topical creams and averaged 4 wk to ulcer healing. They concluded that early dermatologic evaluation should be sought in patients without PPG due to their rapid response to appropriate local therapy[12].

Last *et al* presented their cumulative experience of conservative management in parastomal ulceration in patients with Crohn's disease. They discussed 17 patients with Crohn's disease who developed 28 parastomal ulcers (at least 1.5 cm in diameter), ranging in time from two weeks to seven years after ileostomy construction (the mean time was 45 wk). The methods of management that were utilized included debridement, curettage, unroofing the ulcer complex, pouching of the stoma with Telfa strips in the ulcer base. They reported that most ulcers healed in a mean of 12.7 wk and median of 8 wk. Six patients did not resolve with conservative measures and underwent ileostomy relocation[10]. Last *et al* also suggest that placement of parastomal drains may decrease the rate of infection.

Pyoderma gangrenosum is an idiopathic, inflammatory, ulcerative condition of the skin. A specific type of parastomal ulceration, PPG is unusual and is often misdiagnosed as a stitch abscess, contact dermatitis, irritation from the leaking feces or urine, extension of underlying Crohn's disease, or a wound infection. PPG has been reported in patients with inflammatory bowel disease and occasionally in patients with malignancies. The onset of PPG from the creation of the stoma is extremely variable. Hughes *et al* reported their 7 case experience in a retrospective review in *JAMA*[13]. In these cases, treatment was empiric and the final responses were varied. Histopathology was verified by biopsy. All patients had initially been treated with one or more courses of broad spectrum antibiotics for a presumed skin infection prior to the diagnosis of PPG. Topical clobetasol propionate (a class I topical corticosteroid) used in conjunction with intralesional injection of triamcinolone acetonide was effective in three patients, ineffective in two patients, and used as a possible helpful adjuvant therapy in two others. Three patients required the addition of an immunosuppressive agent (cyclosporine or mycophenolate) due to systemic corticosteroid failure or adverse effects. Oral dapsone also appeared to be helpful in two of the patients. The authors concluded that treatment of patients with PPG is empiric and two fold. The underlying disease process must be treated along with local wound care management (i.e., the addition of a topical corticosteroid)[13].

In children, parastomal excoriation, following chemical irritation can lead to parastomal breakdown and ulceration. Many topical agents have been tried. Hayashi *et al* reported their experience with 15 patients with stomal or perineal skin ulceration treated with topical sucralfate after failure of other common firstline agents. Their results showed complete healing in 13 patents. Their main observations were that noticeable improvement usually took two to three days, healing occurred from the outside in, the sucralfate itself was soothing and improved comfort, when fungal dermatitis was the cause the sucralfate was not effective[14].

The concensus of these studies is that medical management will work for a majority of patients. This includes local wound care, proper appliances, and local and systemic corticosteroids. If systemic disease such as IBD is suspected, the disease should be controlled systemically and the ulcer itself may then resolve. In severe systemic disease, local wound care can be used along with systemic therapy. In many cases it may take weeks for final resolution of the ulcers. The various studies that documented the medical management of parastomal ulcers are summarized in Table 1.

SURGICAL MANAGEMENT

While a last resort, surgical revision of a stoma for parastomal ulceration is a possibility. Reoperation of patients with Crohn's disease is common as a result of stomal complications, including ulceration, and is often associated with recurrence of inflammation both at the ileal site and at the parastomal site[15]. Surgical options include revision of the current stoma or new stoma placement. If the problem is refractory to medical treatment, most surgeons opt to relocate the stoma. The procedure involves takedown of the existing stoma and reformation at a more appropriate site. It is important to optimize systemic disease (IBD) before this is attempted.

Table 1 Medical management of parostomal ulcers

Author	Design	Number of patients	Treatment	Summary
Wolfson *et al*	Retrospective Review	10 patients (8 with IBD), 5 with PPG	Patients with PPG received systemic steroids or IBD therapy. Other patients were treated with conservative measures, corticosteroid creams, wound care.	Patients with IBD should be treated systemically. Patients with other causes for ulceration should be evaluated by dermatology and receive local therapy.
Last *et al*	Retrospective Review	17 patients with Crohn's who developed 28 ulcers	Treatment included debridement, curettage, unroofing the ulcer complex, pouching of the stoma with Telfa strips in the ulcer base.	6 patients did not respond to conservative management, and required relocation.
Hughes *et al*	Retrospective Review	7 patients	Topical clobetasol propionate used with intralesional injection of triamcinolone acetonide in seven patients. Three patients received an immunosuppressive agent (cyclosporine or mycophenolate). Oral dapsone was used in patients.	The underlying disease process must be treated along with local wound care management (i.e. the addition of a topical corticosteroid).
Hayashi *et al*	Retrospective Review	15 children with stomal or perineal skin ulceration	Used topical sucralfate after failure of other common first line agents.	Topical sucralfate can be soothing when other measures fail.

CONCLUSION

Complications with surgically placed ostomies are common and multifactorial. Outcomes can be improved by optimizing the health of the patient, particularly in patients with Crohn's disease or IBD, by careful preoperative planning and placement, thorough surgical training and experience, and by proper wound ostomy nursing care. Unfortunately, despite meticulous preoperative planning, ostomy complications still occur. Parastomal ulceration, although rare, is a particularly difficult management problem. The etiology is varied and treatment possibilities are as well. A review of the literature shows that most patients with parastomal ulcers who do not have associated IBD or PPG respond quickly to local wound care. Patients with PPG, IBD, or other systemic causes of their ulceration need both systemic and local care and are more likely to need long-term treatment, and possibly surgical revision of the ostomy. The treatment is complicated, but improved with the help of ostomy specialists.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Surgical perspectives in gastrointestinal disease: From the editor

In the following 20 articles we have attempted to give an update and overview of different aspects of gastrointestinal disease that are treated via a surgical intervention. In addition we have included topics related to advances and innovations in surgical instrumentation and training that are useful in treating gastrointestinal disease. Additional topics covered deal with the ethics of abdominal organ transplantation, inflammatory bowel disease in adolescents and children, and discussions of the quality of life following gastrointestinal cancer. We have also included a discussion of recent discoveries involving the calcium sensing receptor and how this receptor may be employed as both an alternative therapy as well as a possible preventative measure for a variety of gastrointestinal disease issues. As we hope becomes apparent from this collection of articles that the surgeon has and will remain an integral part of the identification and treatment of gastrointestinal disease. We have also included a topic on the surgical scientist and how the surgeon of the 21st century has become an active player in the bench side investigations of causality for gastrointestinal disease.

It is our hope that this collection of articles will give the reader an in depth understanding of the role of the surgeon in all aspects of patient management; from identification of disease related factors, to the removal or repair of the effected tissue, to the postoperative counseling and management of the patient.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Advances in alimentary tract imaging

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Abstract

Advances in imaging techniques are changing the way radiologists undertake imaging of the gastrointestinal tract and their ability to answer questions posed by surgeons. In this paper we discuss the technological improvements of imaging studies that have occurred in the last few years and how these help to better diagnosing alimentary tract disease.

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Key words: Imaging; Alimentary tract

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INTRODUCTION

Advances in imaging techniques are changing the way radiologists undertake imaging of the gastrointestinal tract and their ability to answer questions posed by surgeons. Technologic advancements in CT have dramatically improved the detection and characterization of a wide variety of intestinal diseases before and after surgery. In the first part of this paper we review some of the advances in imaging technology. In the second part we discuss how these technical advances improve diagnosis of gastrointestinal disease.

ADVANCES IN IMAGING TECHNOLOGY

Multislice computed tomography

CT is now more commonly performed than fluoroscopic studies in analyzing the gastrointestinal tract. Several generations of CT scanners have become available, starting with 4-slice scanners in 1998 to 64-slice scanners in 2005. With each generational advance more rapid scans are possible with better tailored vascular phase acquisition, and thinner slices. When the resolution of the voxels that make the scanned volume is equal in the longitudinal and transverse (axial) dimensions, the study is considered to be isotropic. In an isotropic resolution scan of the abdomen and pelvis the slice width should typically be less than 1 mm. With such scans the reformats in coronal or sagittal planes will have the same image quality as axial images (Figure 1). Using 64-slice scanners, the entire abdomen and pelvis could be scanned in 5-8 s with isotropic resolution. Modern scanners have software that would almost instantaneously create coronal or sagittal reformats. In our practice every abdominopelvic CT is reconstructed routinely in at least the coronal plane in addition to transverse scans. This plane optimizes assessment of organs such as the mesenteric small bowel which have a tortuous and unpredictable course (Figure 2). Three dimensional image sets such as volume rendering may also be created when deemed helpful prior to surgery[1]. Radiologists are gradually moving away from reading axial slices of CT scans to viewing the abdomen as a three dimensional structure, similar to what the surgeon would see during an operative procedure (Figure 3). Several changes to CT techniques have become accepted in the last few years. In the past positive oral contrast, usually dilute iodinated contrast media or dilute barium, were widely used. These were safe and allowed adequate opacification of the small bowel. The current approach is to favor neutral oral contrast with intravenous contrast when there suspicion of small bowel tumor or inflammatory disease[2]. Advantages of this technique include fewer dilution and admixture artifacts with enteric contents, better visualization of enhancing bowel tumors and inflammation, easier processing of CT angiogram and 3D techniques. One disadvantage of orally administered contrast is the reduced distention of the distal small bowel. This disadvantage could be overcome by either infusion of the enteral contrast under hydrostatic pressure using a nasoenteric tube (CT enteroclysis, see below) or by drinking large volumes of contrast, usually more than 2 liters, in predetermined aliquots over a period of time (CT enterography). Water is the most commonly used neutral enteral contrast; other propriety preparations include (VoLumen, EZ-EM, Westbury, NY) which contains agents to reduce absorption and to add flavor.

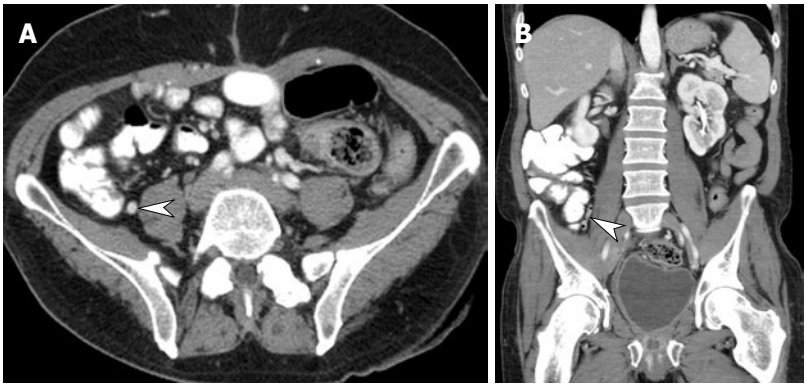


Figure 1 Sixty-three years old man with right lower quadrant abdominal pain. Note the equivalent resolution of the axial image (**A**) and coronal reformat (**B**). In addition it was easier to appreciate the vertically oriented normal appendix (arrowhead) on the coronal reformat.



Figure 2 Abdominal pain and fever in 23 years old female. **A, B**: Coronal reformats with neutral enteral contrast (water) showed dilated proximal loops of small bowel (white solid arrows), and nondistended colon (dashed arrows). A segment of distal ileum (black arrow, image **A**) showed stenosis with mucosal enhancement, consistent with inflammatory stricture causing small bowel obstruction. A second noncontiguous segment of terminal ileum showed intense mucosal enhancement (white arrowhead, image **B**) and comb sign due to mesenteric hypervascularity (black arrowhead, image **B**). The appearances were consistent with acute Crohn's disease. The diagnosis of wall enhancement and thickening is made easier by using water as oral contrast. The coronal perspective also allows easier evaluation of length of involved segments.

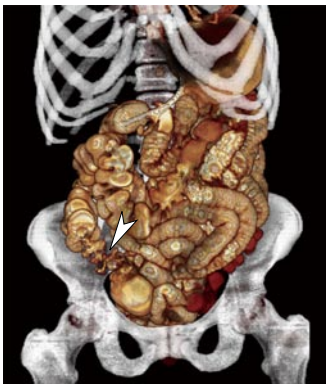


Figure 3 Three dimensional volume rendered image of small and large bowel in 46 years old female with diarrhea. There was no evidence of bowel obstruction. The apparent narrowing of ascending colon (arrowhead) was likely to be due to passage of peristalsis, there was no evidence of mass on two dimensional axial and coronal images. Three dimensional images may be more commonly used in the future to help plan surgery.

CT enteroclysis

CT enteroclysis was first reported in 1992 in an effort to overcome the individual deficiencies of CT and barium enteroclysis and to combine the advantages of both into one technique[3]. In this technique a nasoenteric tube is inserted preferably under conscious sedation and enteric contrast infused under pressure till the entire small bowel is filled. The patient is then taken to a CT scanner to complete the study. As with conventional CT, the choice of enteric contrast is either positive or neutral contrast and depends on the indication[4]. For diagnosing leaks, fistula, peritoneal abscess and low grade small bowel obstruction we prefer positive contrast. For all other indications including assessment of tumors and the inflammatory activity of Crohn's disease we use neutral contrast (Figure 2). CT enteroclysis has several advantages over conventional CT including better assessment of low grade obstruction, enteric fistula, and bowel wall thickening. However this technique has disadvantages of requiring a significant amount of radiologist time, insertion of nasoenteric tube and risks of conscious sedation.

Magnetic resonance enteroclysis

Magnetic resonance enteroclysis is advocated primarily in Europe as a method of interrogating the small bowel without using ionizing radiation. In many ways the technique is similar to CT enteroclysis, except that the nasoenteric tube is inserted in the MRI scanner room prior to the MRI examination. Improvements in magnet technology allow fast scans that could be performed in a reasonable breath-hold. Currently, the poor spatial resolution and motion artifacts impair its value. When compared to CT enteroclysis MR enteroclysis was found to have lower sensitivity and inter-reader agreement in diagnosing a variety of small bowel diseases[5].

CT colonography (virtual colonoscopy)

In this paper we will call CT colonography by its more familiar name, virtual colonoscopy. In this technique very thin slices are obtained in prone and supine positions though the abdomen and pelvis. The resulting images are treated in many ways (Figure 4): (1) Two dimensional reformats in coronal and axial planes; (2) Three dimensional fillet view, where the colon is displayed as if it has been dissected; (3) Endoluminal fly though view. The production of such views requires sophisticated software and appropriate workstation. In our practice, we initially survey the colon on the three dimensional fillet view. Suspected abnormalities are correlated with the axial and coronal two dimensional views, and on the endoluminal view. This approach reduces the time for image interpretation. Measurement of polyp size is made on the endoluminal view, which is considered the most accurate[6]. Subsequently, axial slices are reviewed to diagnose extracolonic abnormalities.

Software programs in routine use include book marking of polyps for correlation with subsequent scans, automatic center line navigation which allows easier fly through, missed patch tool to keep track of surfaces that are not optimally visualized on automatic center line navigation and electronic stool tagging and fluid removal. Currently we prescribe laxatives taken a day prior to the study to reduce the stool burden. However many recent papers have commented on the accuracy of virtual colonoscopy

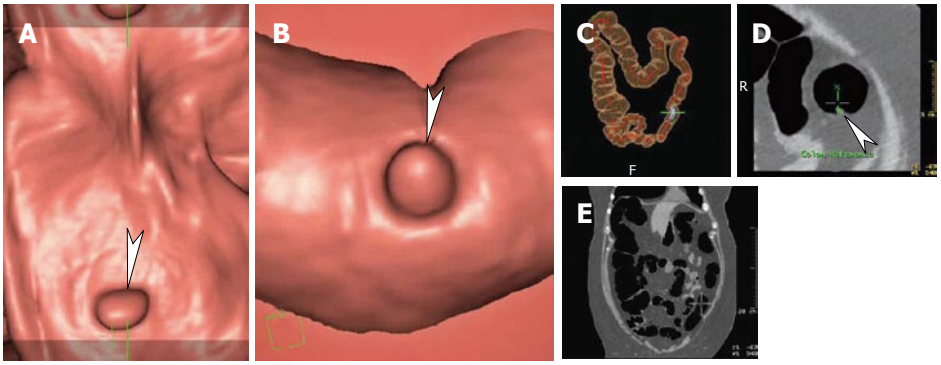


Figure 4 Virtual colonoscopy images in 62 years old female with incomplete optical colonoscopy. **A**: Dissection or fillet view; **B**: Endoluminal fly-through view; **C**: Transparent localizing view; **D**: Two dimensional axial view; **E**: Two dimensional coronal view. At our institution the initial read is the 3 dimensional fillet and endoluminal views in antegrade and retrograde fashion. Sites of concern are correlated with axial or coronal two dimensional views. The entire interpretation including assessment of extracolonic structures takes about 30 min. Note the 6 mm polyp (arrowhead). Its location was confirmed as distal descending colon on image C.

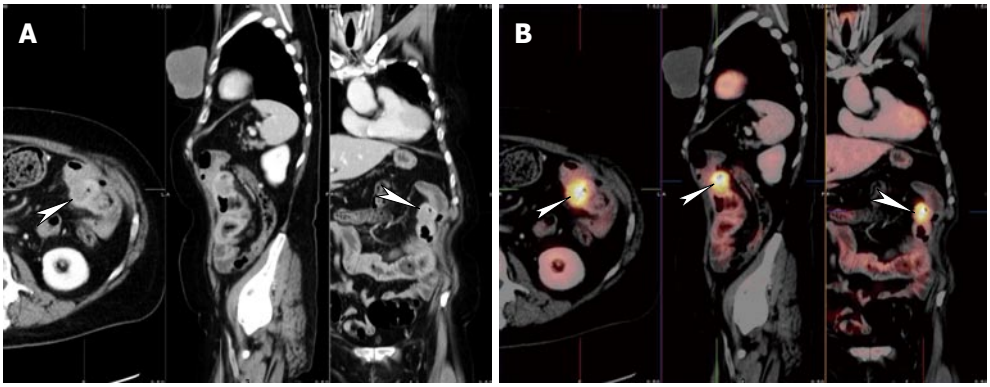


Figure 5 Thirty-nine years old female with prior sigmoid colectomy for cancer, presented with rising CA-125. **A**: Axial, coronal and sagittal images of CT raised concern for possible mass adjacent to the splenic flexure (arrowheads); **B**: Fused images of PET and CT showed hypermetabolic focus (arrowheads) in the mesocolon adjacent to splenic flexure, indicating recurrent tumor. No other sites of tumor were found. While PET is sensitive for recurrent disease it has poor spatial resolution and it would have been difficult to determine if the recurrence was in the colon, mesentery or adjacent spleen. Fusion of PET and CT images allowed accurate anatomical localization.

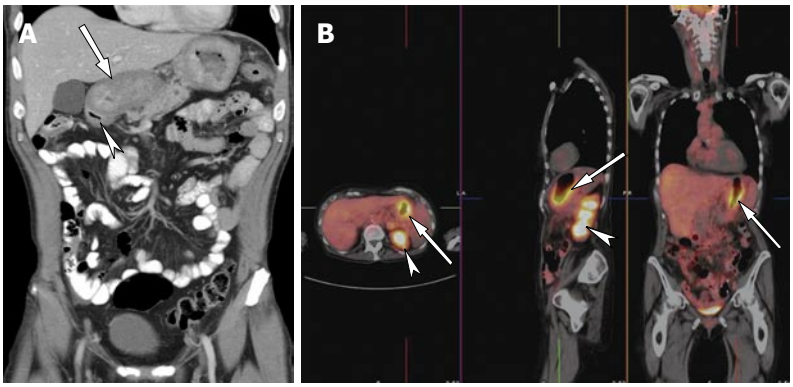


Figure 6 Sixty-nine years old male with dyspepsia. **A**: Coronal reformat showed diffuse thickening of the gastric wall (arrow). The duodenal cap was normal (arrowhead); **B**: Fused PET-CT images showed hypermetabolic activity of the gastric wall indicating cancer. No other foci of abnormal uptake were identified. Note the normal increased activity in the renal collecting systems (arrowheads) due to excretion of radiopharmaceutical.

without using laxatives but instead using low residue diet and tagging feces with dilute contrast[7,8]. The possibility of performing the study without laxative preparation will be a significant advantage of virtual colonoscopy over optical colonoscopy.

Positron emission tomography with CT

Over the past decade positron emission tomography (PET) has changed from a research tool to a full fledged clinical tool excelling in oncological applications. The concept of this technology is based on the fact that tumor cells have a greater avidity for using glucose via glycolysis than most non-cancerous cells[9]. By administering deoxyglucose labeled with a fluorine-18 which is a positron emitting radionuclide (resultant radiopharmaceutical is fluoro-18-deoxyglucose or FDG), it is possible to visualize the location of a tumor. In the first few years of development of PET improvements were made in scanner and detector crystal design. The most significant recent change has been incorporation of PET and CT in the same unit and fusion of the anatomical information from CT and the physiological information from PET (Figure 5)[10]. Both imaging modalities have individual strengths that complement each other. The newer generations of PET-CT scanners utilize multislice CT that allow oral and intravenous contrast information, obviating the need for a separate staging CT scan.

In the gastrointestinal tract PET-CT is used mainly for oncological applications and has been shown to have greater overall accuracy and cost-effectiveness compared to other imaging modalities[11]. PET-CT in the GI tract is commonly used for initial staging, response to therapy and detection of recurrence of esophageal, gastric (Figure 6), colorectal, and gastrointestinal stromal tumors. Additional applications that are showing initial promise include the evaluation of Crohn's disease[12].

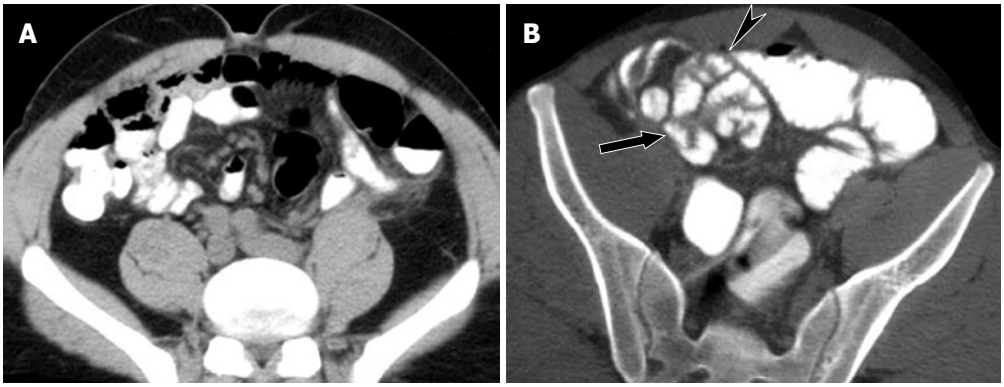


Figure 7 Fifty-four years old with prior hysterectomy presented with abdominal bloating and nausea. **A**: Conventional CT did not show distended bowel or transition point and was interpreted as normal; **B**: CT enteroclysis performed a day later shows distended small bowel loop with beaking (arrowhead) adjacent to anterior parietal peritoneum. Distal small bowel loops were nondistended (arrow). The appearances were of low grade small bowel obstruction due to adhesions.

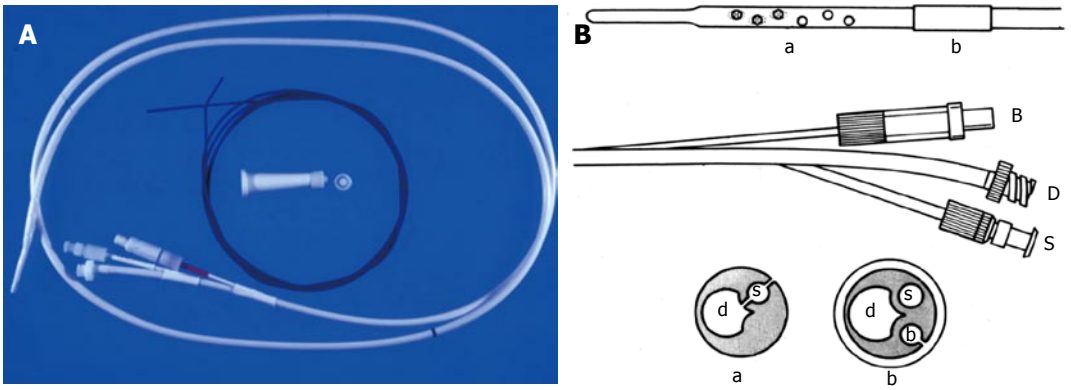


Figure 8 **A**: Image of MDEC decompression and enteroclysis tube with stiffening wire that helps maneuvering the tube into the proximal jejunum; **B**: Line diagram of the tube. B = balloon port, D = drainage port, S = sump port (helps prevent the tube from becoming obstructed by debris). The inset figures show the cross sectional appearances of the tube at positions a and b.

DIAGNOSIS OF MAJOR ALIMENTARY TRACT DISEASE

Gastric tumors

Currently gastric cancer is diagnosed using endoscopic biopsies and locally staged using endoscopic ultrasound. CT is used or more distant staging. Improvements in CT may allow better detection of local invasion of gastric tumor. In a recent study multislice CT had an accuracy of 88.9% in T staging and 70.4% accuracy in N staging, making it comparable to EUS[13]. Virtual gastroscopy, transparency rendering, and 3D mapping of the tumor can help in surgical planning[14]. These techniques however require significant additional time for image creation and interpretation[15].

Small bowel obstruction

Initial studies of conventional CT in small bowel obstruction reported sensitivities of above 90% to 96%, and accuracy of 95%[16,17]. However, these studies were only included patients with high-grade obstruction. In assessing low grade small bowel obstruction, CT enteroclysis is clearly superior to conventional CT (Figure 7)[18]. In addition to identifying the etiology, severity and probable location of small bowel obstruction, CT is useful for determining the presence of closed loop obstruction and strangulation[18]. Recognition of these complications is of great concern to surgeons, particularly those who believe that a trial of conservative nonoperative management is warranted in simple mechanical small bowel obstruction[19]. With the use of multiplanar and 3-dimensional imaging the confidence of accuracy of answering pertinent clinical questions has considerably improved. The development of a multipurpose long tube (MDEC 1400, Cook Inc, Bloomington, IN) has improved the nonsurgical management of uncomplicated partial small bowel obstruction (Figure 8)[20]. Such long tubes have sump ports that are less likely to be occluded and are designed to be used with suction devices currently used in hospitals. Following adequate decompression of the small bowel, a CT enteroclysis may be performed to detect the site and etiology of obstruction.

Crohn's disease

The main groups of Crohn's disease are active inflammatory, fibrostenosing, chronic smoldering and fistulous types[21]. Differentiation of these groups is important in deciding therapy with immunosuppressant, anticytokine drugs and surgery. For instance, inhibitors of the cytokine tumor necrosis factor-alpha have been shown to useful in healing fistulous disease[22] but have no known beneficial effect on, or may exacerbate, fibrostenotic segments. Such drugs are expensive and careful patient selection is required. Multislice CT is quite sensitive in detecting unexpected active Crohn's disease in patients presenting with nonspecific abdominal pain. In determining the severity and extent of known active Crohn's disease neutral oral contrast with intravenous iodinated contrast is the optimal technique. Mucosal hyperenhancement, submucosal edema, wall thickening, and mesenteric hypervascularity ("comb sign") are well demonstrated by this technique (Figure 2). Enteric fistula, fibrostenotic segments and abscess are also diagnosed by this technique. Isotropic imaging with multiplanar reformats is helpful for diagnosis and surgical planning. The important differentiation of small



Figure 9 Neutral enteral contrast CT enteroclysis in 27 years old female presented with chronic diarrhea and anemia. There was a prior history of systemic lupus erythematosis. Coronal reformat showed diffuse smooth small bowel wall (arrow) and fold thickening. Small bowel biopsy showed vasculitis.

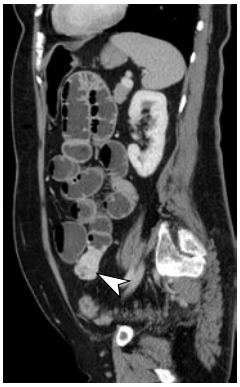


Figure 10 Sagittal reformat of CT enteroclysis in 63 years old female with unexplained gastrointestinal (GI) bleeding showed 3 cm hypervascular submucosal mass (arrow) arising from mid small bowel, proven as a gastrointestinal stromal tumor at surgery. Prior capsule endocsopy had shown no abnormality, other than jejunal angioectasia.

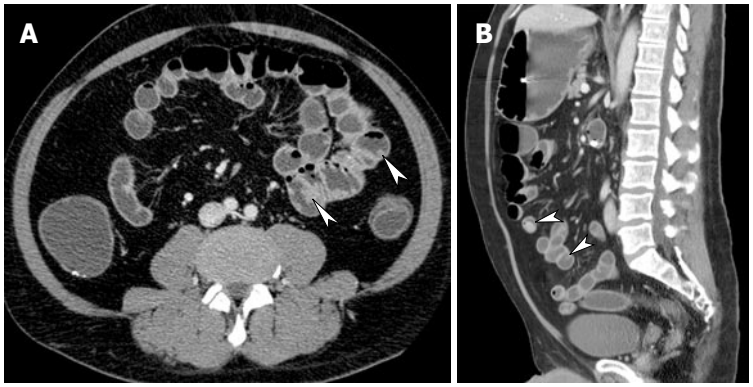


Figure 11 Fifty-two years old male with known Peutz-Jeghers syndrome. Axial (**A**) and sagittal (**B**) images showed multiple polyps of varying sizes in the small bowel (arrowheads). Information regarding the location and sizes of polyps was valuable in performing double balloon endoscopic polypectomy.



Figure 12 Metastatic carcinoid in 59 years old female presenting diarrhea and wheezing. Coronal reformat of isotropic resolution CT enteroclysis performed with neutral enteral contrast showed 3 cm hypervascular mass in distal ileum (arrowhead) and hypervascular liver metastases (arrow).

bowel obstruction due to active inflammation and fibrostenotic disease is accurately performed by CT enteroclysis. Aphthoid lesions of suspected early Crohn's disease, increasingly diagnosed by capsule endoscopy, are usually not visible on CT. If radiological investigation is required in such cases we use barium and carbon dioxide double contrast fluoroscopic enteroclysis. CT is also useful in showing inflammatory small bowel disease other than Crohn's disease (Figure 9).

Small bowel tumors

In the investigation of small bowel mucosal lesions, imaging techniques have had to adapt to the advent of capsule endoscopy. In our institution CT enteroclysis is used to exclude luminal stenosis prior to capsule endoscopy. Where capsule endoscopy is negative despite the high suspicion of tumor, e.g. unexplained gastrointestinal bleeding with positive occult blood, we recommend CT enteroclysis. Capsule endoscopy may miss submucosal lesions (Figure 10). Other reasons for false negative capsule endoscopy include focal lesions at sites where bowel is angulated, where there is rapid or very slow intestinal transit.

The ability to detect polypoid small bowel lesions, as in Peutz-Jeghers, is important when considering double balloon endoscopic polypectomy. In this regard the multiplanar imaging with 16-slice or higher generation CT is useful (Figure 11). In carcinoid tumors the site and number of primary tumors (30% have multiple primary sites) are best diagnosed with CT enteroclysis[23]. Because of its ability to evaluate the small bowel wall and the presence of liver metastases, CT enteroclysis with neutral enteral and intravenous contrast is the preferred technique (Figure 12). Nuclear medicine tests have been used to diagnose and stage carcinoid tumors. Indium-111 or iodine-123 labeled DTPA octreotide, a long acting analogue of somatostatin, has been shown to have a sensitivity of 80%-100% in diagnosing carcinoid[24]. Anatomic localization is improved with the use of single photon emission tomography (SPECT). Fluorine-18 deoxyglucose (FDG) positron emission tomography (PET) may be used for detection of poorly differentiated primary tumors that are not seen with other techniques. However for most small bowel carcinoid tumors, which have slow proliferation, FDG PET is of limited value[24]. PET scanning with fluorine-18 dopa, gallium-68 labeled octreotide or carbon-11 labeled tryptophan has been shown to be very sensitive in localizing tumor extent[24,25]. Small bowel lymphoma is another tumor where FDG PET has been found to be useful. Despite the mild physiologic uptake in the gastrointestinal tract, a positive FDG PET scan after the completion of chemotherapy in patients with small bowel lymphoma is a strong predictor of relapse[26].

Colonic cancer

The application of virtual colonoscopy to colorectal cancer screening caught the attention of the lay and professional communities following a seminal publication[27] which showed that with modern CT technology, virtual colonoscopy compares favorably with optical colonoscopy. Sensitivity of 94% and specificity of 96% for polyps larger than 1 cm were reported[27]. There remain several hurdles before virtual colonoscopy becomes part of the mainstream of colorectal cancer screening methods. These include limited reimbursement by third party payers, size threshold above which suspected colonic polyps will be investigated, ra-

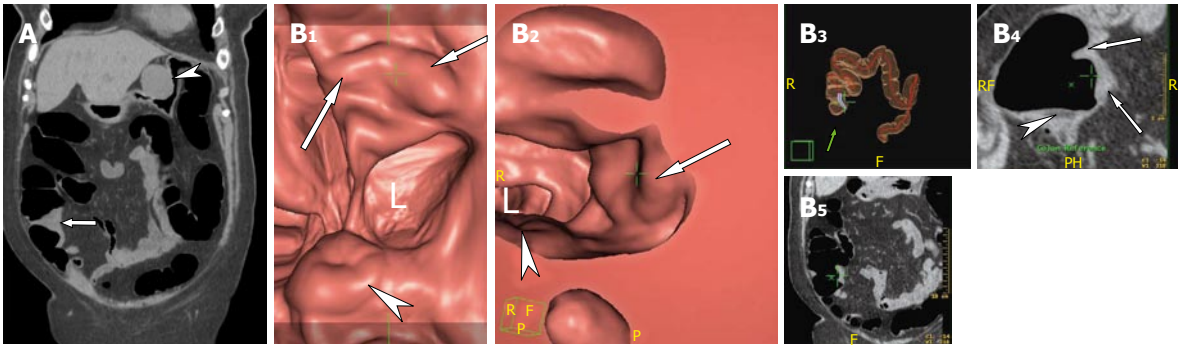


Figure 13 Fifty-eight years old male with incomplete optical colonoscopy. **A**: Coronal reformat showed a mass in the medial right colon (arrow) consistent with colonic cancer. A second mass (arrowhead) showed exophytic growth from the stomach and was subsequently proven to be a gastrointestinal stromal tumor; **B**: Images of virtual colonoscopy showed the mass (arrows) adjacent to the ileocecal valve (arrowheads). L = colonic lumen.

tionale for further work up of incidental findings, such as small low density renal and liver lesions. Currently, virtual colonoscopy is used clinically in patients with incomplete or failed optical colonoscopy (Figure 13) and those with contraindications to sedation. This test has been shown to affect treatment strategy in 7.5 to 19% of patients by identifying additional adenomas and carcinomas[28,29]. The use of virtual colonoscopy in known colon cancer is helpful in evaluating the whole colon for possible missed polyps and stage the cancer at the same time.

In conclusion the practice of alimentary tract imaging will continue to change. Details of the viscera which in the past were only diagnosed by indirect manifestations on barium examination are now demonstrated in exquisite detail by multislice CT. Functional information afforded by PET scanning adds further to the utility of CT in diagnostic imaging. Further developments in 3D imaging for the entire alimentary tube will add to presurgical mapping of many disorders. The modern era of gastrointestinal radiology is here. Active collaboration among surgeons and radiologists will ensure efficient utilization of the various advances discussed in this article.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Controversies in the treatment of common anal problems

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Abstract

Treating common benign anal diseases has evolved towards more outpatient procedures with better outcome. However, minimizing post-procedure morbidities such as pain and the avoidance incontinence remain the most significant concerns. We introduce some controversies and highlight the developments in current surgical practice for the treatment of common anal problems.

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Key words: Anal problems; Treatment

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INTRODUCTION

Benign anal conditions are common and may pose troublesome physical, mental and social ailments to patients. There are numerous surgical techniques and non-surgical treatment used to treat this group of disease and most of the modalities are safe and effective. The main concern of treating anal conditions are the after-treatment undesirable pain and sphincter dysfunction which often pose a significant challenges to the attending physicians.

HEMORRHOIDS

Hemorrhoids develop from engorgement and prolapse of the submucosal anal cushion, which composed of an interlacing arteria-venous hemorrhoidal plexus, supported by connective tissue and minute muscle fibres[1]. The bulk of the anal cushion is located above the dentate line, which is devoid of sensation. The classical positions are described as the left lateral, right anterior and right posterior. The division between internal and external hemorrhoids is by their origin pedicles being proximal (overlaid by mucosa) or distal (overlaid by squamous epithelium) to the dentate line. The pressure within the anal cushion contributes to approximately 15 percent of perfect anal continence[2]. It is reported that continence disturbance co-exists in 40 percent of patients with hemorrhoids[3] and disrupting the anal cushion during hemorrhoid treatment may further worsen it.

The true prevalence of symptomatic hemorrhoids is unknown. It was reported to affect up to 10 million of North American and the European population in 1980s[4]. Predisposing factors include hereditary factors, constipation and increased intra-abdominal pressure, which include pregnancy, ascites, coughing and strenuous work. Inadequate fiber intake, prolonged sitting on the toilet and chronic straining at stool are among the risk factors. The symptoms of hemorrhoids are non-specific and the diagnosis is made by direct visualization via anoscopy or proctoscopy examinations. However, further evaluations using sigmoidoscopy or colonoscopy is useful when symptoms are not truly apparent of hemorrhoids especially in the elderly to rule out more serious conditions such as malignancy[5]. Other common symptoms of hemorrhoids include mass protrusion, pruritis ani, itching, mucus discharge and perianal pain[3,5]. Intermittent mass protrusion and occasional painless bleeding often do not require urgent treatment but acute symptoms of pain, bleeding and incarcerated protrusion warrant immediate action. In practice, the classification of hemorrhoids generally follows their prolapsing characteristics and they are staged into 1-1V degrees as outlined by Goligher. This classification serves as a useful guide towards choice of treatment modalities. However, comparison of treatment efficacy is largely focused on symptomatic relief and satisfaction outcome that are mostly measured using pain scores, recurrence rates and disturbance of continence after treatment.

Non-surgical treatments are indeed convenient and can avoid unnecessary complications of surgery. Unfortunately, many of these are lacking in evaluation to confirm their role in hemorrhoid treatment. High fiber intake and laxatives are employed in almost all hemorrhoid stages as an initial choice of treatment with the intention to prevent constipation and straining at defecation and reduces local

inflammation. Similarly, antidiarrhea agents are used when diarrhea or frequent defecation exacerbate hemorrhoid symptoms. These have temporarily reduced hemorrhoids symptoms as shown by several placebo-control studies[6-9]. Relief of symptoms is further enhanced with emphasis on anal hygiene. In general, the symptomatic resolution using conservative measures alone might be temporary and more patients seemed to need further surgical intervention after conservative treatment. Numerous ointments, creams and suppositories are being used as topical agents for hemorrhoids. They are mainly composed of local anesthetics, steroids and several skin irritation preparations to ameliorate symptoms of prurits ani or pain. Their function as remitting agents is indeed very limited.

The most known oral pharmaceutical agent used to treat hemorrhoids is Daflon™, which consists of mainly micronized diosmin and hesperidin. This is a venotropic compound used in various venous disorders. A dose of 500mg taken two to three times daily rapidly diminishes variable degree of hemorrhoid symptoms such as pain, bleeding and swelling within 10 d of treatment[10,11]. In addition, an extended dosage studied by Godeberge[12] using Daflon 1000 mg bid for two months has resulted in 60 percent symptoms severity reduction as compared to 30 percent of the placebo group without much side effects. Nevertheless, rapid symptomatic relief alone might not be satisfying and it is still unclear concerning the ability of this oral pharmaceutical agent in preventing hemorrhoids surgery. Daflon™ is however not readily available in the United States and the use of any pharmaceutical agents for hemorrhoid treatment has yet to achieve wide acceptance among North American physicians.

Asymptomatic external hemorrhoids do not require surgical intervention but these are prone to thrombosis. The treatment for thrombosed external hemorrhoids is rather straightforward. The main aim is to rapidly eliminate pain by means of general conservative measures, clot evacuation or surgical excisions. The choice of treatment depends largely on the severity of pain. It will be wise to embark on surgical procedures at the peak of the pain which is within 24 to 72 h of its initiation. This is because the procedures themselves might be more painful. When the pain is somewhat tolerable, conservative measures is useful because most pain decreases with time. However, surgical excision is more effective with lower relapse rate and longer remission intervals than conservative measures[13]. On the other hand; the treatment for symptomatic internal hemorrhoids had undergone numerous revolutionary modalities. Multiple ablative techniques had been used and these are mainly aimed at fixing of the anal cushion and disrupting the hemorrhoid circulation by means of local office procedures or surgical excision (hemorrhoidectomy). Among the office procedures are injection sclerotherapy, rubber band ligation, diathermy, cryotherapy and infrared photocoagulation. Most of these are simple to perform and capable of producing satisfying short and long-term improvement. Furthermore, repeated attempts can be done until satisfying results achieved. The overall success rates measured by symptomatic improvement and avoidance of further intervention were 80 percent to 90 percent[14-16]. Controversies remain with regard to the best procedure that produce the least complications of pain, bleeding and incontinence. These complications are mainly technical and frequently used as gauge for patients' satisfaction between treatment modalities. Rubber band ligation seemed to have better long term efficacy in terms of lower recurrence and complication rates than other modalities in treating second and third degree hemorrhoids[17-21]. These complications can be substantial and may affect overall treatment success[20-22]. There were many comparison studies between two modalities and the results did not show clear advantage of any procedure over another. Initial results from the use of rubber band ligation for second and third degree hemorrhoids has shown better long term efficacy especially in reducing prolapse but complications such as pain and bleeding remained the same[17-21]. However, in a meta-analysis, Johanson *et al*[22] found that the curative rate was almost similar for all methods of treatment after 12-mo follow-up. The rubber band ligation had the best result at preventing recurrence but complication of post treatment pain was the least after infrared coagulation treatment. Gupta[23] also demonstrated similar result in a randomized study comparing rubber band ligation and infrared coagulation treatments. Combining two modalities has also produced good therapeutic result. Chew *et al* combined injection sclerotherapy with rubber band ligation and achieved 90 percent cure or improvement. The complication rate was 3.1 percent with an overall recurrence rate of 16 percent. Only 7.7 percent of these patients required hemorrhoidectomy[24]. In summary, these studies have shown that most of the office treatments are safe and effective for low grade hemorrhoids but the best among all is yet to be confirmed. Correcting technical issues has lowered the complications rates making office treatment for first to third degree hemorrhoids tolerable and satisfying.

Hemorrhoidectomy is currently the ultimate treatment for advanced or severe hemorrhoids. It has been the standard and eventual treatment for decades before the introduction of stapled hemorrhoidopexy in 1997. There are numerous techniques of hemorrhoidectomy but the two most popularly used are the Milligan-Morgan open procedure and the Ferguson closed procedures. The Milligan-Morgan procedure involves excision of the hemorrhoids with the overlying mucocutaneous tissue. The excision will need to preserve adequate skin bridges in between excised hemorrhoids to prevent stricture. After achievement of hemostasis, the wound is left opened with a classical clover appearance for secondary healing. In the Ferguson procedure instead, the wound is sutured together for healing by primary intention using absorbable materials. Both these techniques had achieved up to 90 percent cure for third and fourth degree hemorrhoids with few wound complications[25-27]. These operations are associated with significant post operative pain and the exact etiology is still unknown. It is thought to be due to anal sphincter spasm and concurrent bacterial infection. The use of local anesthetic injection or peripheral nerve block has shown to benefit patients in terms of immediate pain control[28,29]. These can be combined with oral analgesics and antibiotics such as Metronidazole[30] to alleviate pain. In addition, a con-

comitant lateral internal sphincterotomy was also shown to reduce pain without causing more incontinence[31]. However, routine usage of perioperative antibiotics and lateral internal sphincteromy to diminish postoperative pain are controversial.

Another commonly encountered problem of hemorrhoidectomy is bleeding. There are several modifications of the classical technique have been tried to overcome it. This involved using diathermy, laser, Ligasure™ and Harmonic scalpel™ during hemorrhoid excision. Other complications of hemorrhoidectomy include temporary urinary retention and wound infection, which are usually treated by conservative measures. In 1997, Longo introduced the treatment of prolapse hemorrhoid by means of a stapled mucosectomy. It is indeed the only method that does not require the hemorrhoids to be excised and the term 'stapled hemorrhoidopexy' was hence used[32]. It is otherwise known as the procedure for prolapse hemorrhoids (PPH) and involves excision of rectal mucosa just proximal to the dentate line and fixing the two cut end together using a circular staple device. This results in disruption of hemorrhoidal circulation and produces healing. Stapled hemorrhoidopexy have more peri-operative advantages and seems to fair equal if not better to hemorrhoidectomy in terms of patients' tolerability. However, its effectiveness as an ultimate treatment for prolapse hemorrhoids awaits further evaluations. Comparison with standard excision hemorrhoidectomies in several randomized trials has shown that stapled hemorrhoidopexy produced shorter hospital stay and better tolerated especially during the immediate post-operative period. It also has the advantage of providing earlier return to normal daily activity for patients[33-40]. This may be largely due to the much reduced pain resulted from the procedure. The major short and long-term complications of stapled hemorrhoidopexy were also rare and comparable to the conventional hemorrhoidectomies. Apart from pain, the complications observed include temporary incontinence disturbance. The theoretical anal sphincter trauma or injury caused by instrumentation of this technique did not seem to be real as shown by studies that assessed anal sphincter function and anal manometry at follow up[33,34,39]. Relapse of symptoms occurred in less than 5 percent of cases with an average of 90 percent patients satisfaction can be achieved. This however, did not differ from the hemorrhoidectomy procedures. Most relapse cases in those trials were successfully salvaged using local office treatments or hemorrhoidectomies. In a most recent study by Gravie *et al*, stapled hemorrhoidopexy had produced good long-term outcome of 85 percent cure after 2 years with comparable morbidity to the conventional hemorhoidectomy[41]. This result was shown to be reproducible by any level of operators.

In summary, patients' comfort and avoidance of complications are the prime issues in treating symptomatic hemorrhoids. For optimal outcome, careful symptomatic assessment in determining the degree of disease severity is an important step for the physician to decide which modality suits their patients best.

ANORECTAL ABSCESS

Anorectal abscess is a common condition caused by infection of the anal glands situated in the crypt of the anorectal junction. The spread of the infection into the intersphincteric space resulted in collection of pus into surrounding perianal spaces. It commonly affects young men with the common symptoms include perianal pain, fever, tender lump around the anus and sometimes produces discharge and incontinence[42,43]. In practice, the Park's classification of anorectal abscess into perianal, intersphincteric, ischio-rectal and supralevator is widely used. Perianal and intersphinteric abscesses occurred more commonly then the deeper ischiorectal and supralevator abscesses[42-44]. Apart from the cryptoglandular origin; causes of anorectal abscesses include Crohn's disease, tuberculosis, malignancies and trauma. Patients who are diabetic and immunosuppressed are also prone to ano-rectal abscesses.

Small and simple perianal and intersphincteric abscesses are usually cured by adequate drainage, which is done through a skin incision or transrectally. Clinical examination under anesthesia frequently provides accurate assessment. For the more obscured abscesses, radiological guidance such as CT scan may help in delineating severity.

A perianal eliptical radial incision over fluctuant area of the abscess and breaking of all locules are sufficient to eliminate infection. The incision is preferred to be as close possible to the anus so that any concomitant fistula can easily be treated later. Specimen for culture and sensitivity is not necessary because antibiotic is not needed for treatment. The use of antibiotics is only for extensive cellulitis, necrotizing lesions or in immunocompromised patients or those with valvular heart disease or implants. However, culture result differentiating gut from skin flora is rather sensitive in determining the presence of fistula[42-47] although this is no better than the surgeons' careful search[48]. All necrotic tissues are minimally debrided with care not to injure the anal sphincters and often drains are used to facilitate drainage. The wound is left opened for secondary healing along with adequate analgesia and sitz bath. Reported complication and abscess recurrence were low at 3 and 0.6 percent respectively[42] and the reasons for recurrence were mainly inadequate drainage or missing a concomitant fistula[49]. The treatment of anorectal abscess is more complex when it is deep, large, multiple or associated with a fistula. These may occur due to the abundant adipose tissue filling the supralevator and ischiorectal spaces. In addition, a horseshoe abscess may develop from deep post anal space abscess connecting to the ischiorectal spaces. In these abscesses, the main concern is the presence of concomitant fistulas. The reported incidence is 35-65 percents[43,50].

Beside draining and debridement, the treatment of the abscesses requires meticulous search of the internal openings and determining the amount of anal sphincters involvement along the fistulous tracks. This can be difficult in the setting of active acute inflammation. Preoperative endoanal ultrasonography and magnetic resonance imaging may facilitate in delineating the complexity of these.

In most instances, the detection of an internal opening is achieved by passage of pus seen at proctoscopy and gentle massaging of surrounding abscess swelling. Once identified, the decision to drain percutaneously or transrectally is much based on the type of abscess that one deals with. This is to avoid the development of high trans-sphincteric or extrasphincetric fistulas, which may develop from drainage of supralevator and ischiorectal abscesses. The drainage of supralevator abscesses need special attention to its aetiology[44]. It may be associated with both pelvic inflammatory process or trans-sphicteric spread of cryptoglandular origin. Drainage through the rectum is preferable when they originated from intersphincteric spread. This is to avoid forming a suprasphincteric fistula which is much more difficult to treat in later dates. Another important consideration is to perform fistulotomy or fistulectomy during abscess drainage or to do staged procedures. Excessive sphincterotomy produces incontinence due to inability to judge precisely the amount sphincter involved. In one prospective study, Schouten *et al*[51] compared patients who underwent concomitant fistulectomy from those who had a staged drainage with secondary fistulectomy. They found that immediate fistulectomy at drainage is associated with 40 percent continence disturbance which tend to worsen overtime. They then recommended for abscesses to be drain adequately first and fistulectomy is reserved for a second stage procedure. In contrast, others have demonstrated that concomitant fistulectomy is safe and effective in eliminating fistula and prevent recurrence[50,52]. Persistent symptomatic fistula occurred in 25 percent of patients who did not undergo primary fistulectomy and incontinence were similar whether or not a primary fistulectomy is performed. However, in acute setting of abscess, the use of stage procedure using drainage seton will potentially minimize anal sphincter injury with fistulotomy or fistulectomy.

PERIANAL FISTULA

Perianal fistula and ano-rectal abscess are merely two components of a disease encountered at a different time. Typical symptoms of their co-existence are recurrent perianal pain with chronic purulent discharge. Common etiological factors denote similar therapeutic approach and the presence of one component will merit the search for the other for a complete cure. The Park's classification[53] of perianal fistula into intersphincteric, transphincteric, suprasphincteric and extrasphincteric described the anatomical tract route in relation to the anal sphincter complex, which is useful for therapeutic guidance. Division into simple and complex fistulas entails risk of sphincter dysfunction upon treatment. The main goal of treating perianal fistulae is to eradicate sepsis and eliminate epithealized tracks without causing incontinence. Patients' satisfaction after fistula treatment is largely related to recurrence and the effect on continence[54].

For the low and simple fistulas, effective treatment is by cutting open the fistulous track (fistulotomy) for secondary healing to take place. It is easy to perform but meticulous assessment must be emphasized on the amount of external sphincter involvement especially in multiparous women. Excising the entire fistula track (fistulectomy) when minimal anal sphincter is involved is also safe but has to be performed with precision. The overall rate of recurrence by these procedures is low at around 4 percent[55,56]. Despite this, the reported incontinence rate was up to 45 percent. This mainly occurred after fistulectomy, which also gave a longer healing time due to the larger tissue excision. Risks factors for incontinence include anal deformities from previous surgery, prior diarrhea and internal sphincterotomy[55,57].

The treatment for complex and high fistulas involving multiple tracks and abscesses needs more diligent strategy. Traditionally, if more than half of the sphincters are involved a staged procedure with cutting setons is used. However, identification of tracks in complex fistulas requires a combination of careful clinical and perhaps radiological assessments prior to intervention. Hydrogen peroxide enhanced endorectal ultrasound and magnetic resonance imaging may aid in delineating the tracks and abscesses[58-60]. These examinations will also allow assessment of anal sphincter anatomy and tone prior to surgery and exclude fistulas from Crohn's disease, perianal tuberculosis and malignancy. Guided blunt probing of the tracks will enable drainage seton insertions for abscess and identification of external anal sphincter for later interventions. When the sepsis is eliminated, a cutting seton which is changed or tightened in the office at certain follow up intervals can be applied. The cutting seton will cut through the muscle producing immediate fibrosing effect that prevent sphincter retraction. Using this method, the healing rate is high and recurrence of fistulas occurred at only 4-6 percent of cases[61-63]. However, cutting setons are associated with 19 to 63 percent of incontinence and causes pain and discomfort. The reason for incontinence is multifactor. While it appears obvious that excessive cutting of the external anal sphincter might be the main reason for incontinence, preserving the internal component of the sphincters may be advantages. A technique introduced by Zbar *et al*[64] had shown that by excluding the internal anal sphincter using a modified cutting seton technique gave better continence result by virtue of better post-operative anal pressure measurements. The technique combined a short mucosal flap (not incorporating the IAS musculature) to close the internal opening, and IAS repair and a re-routing of seton (for EAS cutting) through the intersphincteric space. In essence, identification of patients who are prone to develop incontinence after a cutting seton may help. This includes those who had multiple abscess drainage or previous fistula repairs and patients with chronic disease or infection[61]. Avoiding excessive sphincter cut in these patients would certainly be wise. An alternative to overcome cutting seton morbidities is to use a two stage seton fistulotomy. This involved the insertion of a loose seton for abscess drainage followed by a scheduled secondary fistulotomy. The seton could be left for long period of time until sepsis settles or satisfactory healing achieved especially in complicated cases like Crohn's disease[65].

Another option of treating perianal fistulas is using

the advancement flap procedures. This involves elevating flaps composing of rectal mucosa, submucosa and internal sphincter to cover the internal opening without tension. Associated sepsis is initially eradicated and this procedure will require a good bowel preparation to achieve better healing. It is mainly performed when fistulotomy is either dangerous or difficult such in high trans-sphincteric and suprasphincteric fistulas, the anterior fistulas and fistulas associated with Crohn's[66-70] In addition, advancement flap procedures are particularly useful in patients who had failed previous treatment[68,69]. The effective healing rate is between 60-82 percent[68-72]. It is expectedly associated with low continence disturbance of less then 10 percent but the recurrence rate varies from 8 to 40 percent[70-72]. Recurrence and failure are especially observed among patients with Crohn's disease or those who had multiple prior fistula procedures[70-72].

Another method in treating perianal fistula is the use of fibrin glue, which obliterates fistula opening and tracks. This method avoids the extensive dissection of other techniques especially in complex fistulas. The reported success rates for these complex fistulas were moderate and vary from 14 to 60 percents[73-75]. Its use is potentially good in short tract and low-lying fistulas and theoretical continence disturbance from fibrin glue use in these should not be real although reports regarding it are not available. For highlocated internal openings and more proximal fistula, fibrin glue application may be performed using endoscopic assistance[74,75].

PERIANAL CROHN'S DISEASE

In Crohn's disease prevalence of perianal lesions is about 6 to 29.6 percent and it is associated with more colonic and than ileal diseases[76,77]. Severe perianal disease with typical features of multiple non-midline fissures and fistula lesions is rather easy to recognize but it is often difficult to diagnose perianal Crohn's from fistulas of cryptoglandular origin with confidence without active intestinal disease. Furthermore, the yield of histological granuloma for definitive diagnosis is only at 20 to 40 percent of biopsies[78,79]. It is therefore challenging to decide on anti-cytokine therapy or surgical treatment without confirmatory diagnosis. In addition, even when the diagnosis is obvious, healing of perianal lesions from medical treatment is very unpredictable resulting in recurrent attacks and intractable disease. Infliximab, which is an anti tumor necrosis factor antibodies, provides a 60-70 percent response rate in fistulizing Crohn's disease patients who were refractory to immunosuppresant therapy[80,81] and the best response was achieved among the patients with perianal disease[81,82]. It has thus prevent a significant proportion of patients from surgery because excessive radical surgery to treat perianal Crohn's disease may worsen the existing condition. Traditionally, proctectomy and ileostomy is used to treat severe perianal Crohn's disease. Current efforts are now focused on interventions that will alleviate symptoms and avoid proctectomy. More sphincter sparing and conservative measures such as long duration setons were used effectively[83,84]. Fistulotomy is reserved for superficial fistulas and endorectal advancement flap surgery may be appropriate for more complex fistulas after sepsis eradication in patients with permissible rectal disease, competent sphincters and absence of strictures[85,86].

ANAL FISSURE

Comprehensive systematic reviews with regards to the treatment of anal fissures are readily available[87,88] and this chapter is indeed containing a brief overall management summary. Anal fissure is a break in the squamous epithelia of the anal canal. Its true etiology is not completely understood but hypertonic internal anal sphincter is a common finding and this is thought to cause ischaemia and ulceration. However, cases of anal fissure with normal or even lower resting anal pressure is not uncommon. Ninety percent of fissures are found in the midline over the posterior part of the anal canal and it is prevalent in young individuals of the third and fourth decade. Associated anal conditions include Crohn's disease, hemorrhoids and perianal fistula. Typical symptoms of anal fissures are excruciating pain at defecation, which sometimes followed by post defecation bleeding and pruritis ani.

The division of acute and chronic anal fissure is mainly by the length of symptoms existence. Persistent symptoms of 6 wk or more preclude chronic fissures. In addition, an acute anal fissure has sharp margins with signs of acute inflammation and pink granulation tissue overlies the base while the chronic fissures have raised margins and lack of granulation. Often, horizontal fibers of the internal anal sphincter, which is sometimes hypertrophied, can be seen at the base of a chronic anal fissure. The diagnosis of anal fissure requires direct examination of the anal canal usually performed under anesthesia. Anal manometry and continence score are the commonly used tools to accurately assess treatment outcome since the reduction of anal resting and squeeze pressures correlate well with symptoms improvement and healing. Disturbance of continence is indeed the main complication found in every treatment modality. Most acute fissures will resolve after conservative measures of regular fiber diet modification, stool softener and topical and oral analgesia. This will also cure 10% of chronic fissures but the remaining chronic fissures will require further interventions. The use of topical pharmaceutical agents (chemical internal sphincterotomy) is largely aimed at reducing anal sphincter hypertonia which will then reduces ischaemia and hence promote fissure healing. Topical glyceryl trinitrate (GTN) has a symptomatic and healing effect on chronic anal fissures by causing transient relaxation of anal sphincter through exogenous nitrous oxide provision. Healing is usually observed after a period of 8 wk applications. Studies that attempted to determine the optimal dosage for GTN has been eluded by the high improvement rates shown in the placebo arms due to sampling that might include patients with acute fissures[89-91]. In a study by Thornton *et al*[92], rapid reduction in mean anal resting pressure by 20 percent after first application was achieved. For the patients that were compliant, 73 percent of them achieved symptoms relief with 64 percent fissures healed after 8 wk treatment. The clinical improvement was

also found to correlate positively with high pre-treatment maximum anal resting pressure in the mid anal canal and lower fissure score. The main side effect of topical GTN is headaches that occurred in 20-30 percent of treatment and this was independent of the dosage used despite producing 46-50 percent healing[89-91].

Another pharmaceutical group of substance used to treat chronic anal fissures were the calcium antagonist. Topical nifedipine produced up to 90 percent healing and was better in terms of headache complication compared to GTN[93,94] In another study, calcium antagonist diltiazem produced significant pain and bleeding reduction in an additional 48 percent of patients who had either failed GTN treatment or those who could not comply to GTN due to severe headaches with lesser headaches[95].

Botulinum toxin injected around the fissure and the anal sphincters is another substance used in treating chronic anal fissure. It is also believed that the mechanism involved is related local production of nitrous oxide resulting in sphincter relaxation even though the exact mechanism is still obscured. In one double-blind placebo controlled trial of patients who had failed GTN topical treatment, Maria *et al* demonstrated that injection of 20 U of botulinum toxin produced symptoms relief and healing of 86 and 53 percent respectively at one month follow-up as compared to 13 and 27 percent in the placebo group[96].

Furthermore, the healing rate improved after 2 mo follow up and repeat injection can be performed to avoid surgery. It was also not associated with severe local or systemic complication except for transient incontinence, which tends to recover expectantly[96,97]. However, after longer follow up period, it was reported that more than half of patients who received botulinum toxin injection for chronic fissures required lateral internal sphincterotomy due recurrence[98,99].

Currently, the best available treatment for chronic anal fissure is lateral internal sphincterotomy (LIS). It is done by dividing the distal one third of the internal sphincter fibers without extending beyond the dentate line. The sphincter is divided over the lateral aspect to ease healing and management after surgery. Comparison with topical chemical use had repeatedly shown LIS is a better modality with persistent healing rates of 90 percent and provides more rapid symptoms improvement[100,101]. It also produces high patients satisfaction and better quality of life[102]. The reported continence disturbance after LIS is between 10-30 percent and severe incontinence to solid stool occurred in 1 to 3 percent of treated patients[100-103]. Recently**,** efforts are being made to avoid incontinence using a tailored[104] or controlled[105] techniques. These involve precise graduated division of the sphincter tailored to the fissure length rather than the dentate line as the reference points. These techniques had claimed fewer incontinence and stenosis complications. Covering an anal fissure with a skin flap by means of a V-Y flap or a rotational flap is another surgical modality available for chronic anal fissure. This is probably the best method for recurrent fissures after surgical treatment or traumatic sphincters or patients with weak sphincters (low pressure fissures) such that occur in multiparous women or previously irradiated patients. The technique provides the advantage of avoiding further sphincter injury. Unfortunately, reported series comprised small number of patients and it is rather difficult to make conclusions with regards to its effectiveness[106-108]. These techniques seemed to be easy to perform and maybe considered as an alternative methods in treating chronic anal fissures.

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TOPIC HIGHLIGHT

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Controversies in the treatment of gastroesophageal reflux and achalasia

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Abstract

The immense success of laparoscopic surgery as an effective treatment of gastroesophageal reflux disease (GERD) and achalasia has established minimal invasive surgery as the gold standard for these two conditions with lower morbidity and mortality, shorter hospital stay, faster convalescence, and less postoperative pain. One controversy in the treatment of GERD evolves around laparoscopic antireflux surgery (LARS) as the preferred treatment for Barrett's esophagus and the procedure's potential to reduce the risk of adenocarcinoma of the esophagus. GERD has also been associated with respiratory symptoms, asthma and laryngeal injury, and a second controversy prompts discussions about whether total or partial fundoplication is the more appropriate treatment for GERD. A new and promising alternative in the treatment of GERD is endoluminal therapy. Three types of this new treatment option will be discussed: radiofrequency energy delivered to the lower esophageal sphincter, the creation of a mechanical barrier at the gastroesophageal junction, and the direct endoscopic tightening of the lower esophageal sphincter.

Laparoscopic surgery is discussed not only as a very effective treatment for GERD but also as permanent cure for achalasia. This review analyzes the three most important treatment options for achalasia: medications, pneumatic dilatation, and surgical therapy. Medications as the only true non-invasive option in the treatment of achalasia are not as effective as LARS because of their short half-life and variable absorption due to the poor esophageal emptying. The second treatment option, pneumatic dilatation, involves the stretching of the lower esophagus and is still considered the most effective non-surgical treatment for achalasia. Finally, surgical therapy for achalasia and the two major controversies concerning this laparoscopic treatment are discussed. The first involves the extent to which the myotomy is extended onto the stomach, and the second concerns the necessity and type of antireflux procedure to prevent GERD after myotomy.

LARS and laparoscopic Heller myotomy are the agreed upon as the gold standards for surgical treatment of GERD and achalasia, respectively. In the hands of an experienced laparoscopic surgeon both are safe and effective treatments for patients with excellent subjective and objective long-term results with at least 90% patient satisfaction.

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Key words: Gastroesophageal reflux disease; Nissen; Toupet; Fundoplication; Achalasia; Heller myotomy

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INTRODUCTION

The immense success of laparoscopic surgery as an effective treatment of gastroesophageal reflux disease (GERD) and achalasia has established minimal invasive surgery as the gold standard in the surgical treatment of these two conditions. Compared to open surgery, laparoscopic procedures result in lower morbidity and mortality, shorter hospital stay, faster convalescence and less postoperative pain. The effectiveness of minimally invasive therapy has increased the surgeon's involvement in the diagnosis and treatment of patients suffering from GERD or achalasia and has also resulted in a more profound understanding of the pathophysiology of these diseases.

GERD

In 1956, Nissen[1] was the first surgeon to treat severe GERD by wrapping the gastric fundus around the distal esophagus. The Nissen fundoplication rapidly became the standard for the surgical management of GERD.

Dallemagne *et al*[2] performed the first laparoscopic Nissen fundoplication in Belgium in 1991. After quickly

establishing the safety and efficacy of the procedure, laparoscopic antireflux surgery (LARS) became the state of the art surgical treatment for GERD.

With increased experience and improvements of advanced laparoscopic and esophageal surgical skills, there has been a significant decline in early and long-term complications. In a review of more than 10 000 reported laparoscopic antireflux procedures, morbidity was uncommon (6%), mortality was rare (0.08%), and the reoperation rate was low (4%)[3]. The overall mortality rate is generally low, but does depend on the experience of the surgeon. The rate decreases from 1.3% with an experience of less than 5 cases, to 0.0% with 50 cases or more[4]. The most common side effects of LARS are dysphagia and bloating, but these symptoms are usually self-limited. Dysphagia, for example, is common in the immediate post-operative period, but usually resolves in the first six weeks after surgery; persistent dysphagia occurs in only 2% to 5% of patients[5]. The symptom of bloating also seems overemphasized as a surgical side effect because the prevalence of bloating is quite similar in both surgically and medically treated patients[6].

Although the ultimate goal of both medical and surgical therapy is the alleviation of symptoms, surgical therapy imparts a mechanical solution to GERD whereas medical therapy is aimed at acid suppression. Despite the disparate mechanisms of action, comparisons of medical and surgical therapy of GERD enliven gastroenterologists and surgeons alike. Additional debate concerns the effectiveness of LARS to reverse reflux related injury (e.g., Barrett's esophagus) or alleviate chronic respiratory disease. Finally, technical considerations of LARS, such as the utility of partial or complete fundoplication or the necessity to divide the short gastric vessels, have yet to be completely reconciled.

Medication vs operation

Antireflux surgery has been shown to be very effective at alleviating symptoms in 88% to 95% of patients, with excellent patient satisfaction, in both short and long term studies[7-9]. These excellent results include patients with complicated GERD, such as those with large hiatal hernias, refractory esophagitis and peptic strictures. In addition to symptomatic improvement, the effectiveness of LARS has been objectively confirmed with 24-h pH monitoring, which clearly demonstrates excellent control of esophageal acid exposure more than five years after surgery[8]. When compared to medical therapy, surgical therapy has proven to be superior. A randomized, multicenter trial, with 5-year follow-up, demonstrated that antireflux surgery (open fundoplication) is more effective than proton pump inhibition (PPI) in controlling gastroesophageal reflux disease symptoms[7,10].

Another randomized trial, with 10 year follow-up, evaluating the effectiveness of medical therapy (omeprazole) versus antireflux surgery found that patients who underwent surgery had improved relief of symptoms when compared to the medically treated group[11]. Proponents of medical therapy argue that 60% of patients in the surgical group were taking antacids on a regular basis and should therefore be considered treatment failures. However, the liberal use of antacids in medical practice today makes medication use an unreliable outcome measure of LARS. This is supported by the fact that up to 75% of patients who take such medications after Nissen fundoplication have normal 24-h pH results when tested[12].

Barrett's esophagus

Barrett's esophagus, defined microscopically as intestinal metaplasia of the lower esophageal epithelium, is a result of severe GERD, and carries with it an increased risk of progression to adenocarcinoma of the esophagus. Many patients with Barrett's epithelium have GERD symptoms that are difficult to control with medical therapy, and there is little evidence that the natural history of Barrett's can be affected by proton pump inhibitors (PPIs). Oelschlager *et al*[13] recently reported excellent control of reflux and associated symptoms after LARS in patients with Barrett's esophagus. Complete regression was demonstrated in 34 of 106 (32%) patients with intestinal metaplasia, and in 55% of those with short segments (< 3 cm) of Barrett's esophagus. Hofstetter *et al*[14] observed that low-grade dysplasia regressed to nondysplastic Barrett's in 7 of 16 (44%) patients, and in 9 of 63 (14%) intestinal metaplasia regressed to cardiac mucosa.

Additionally, Bowers *et al*[15] reported a regression rate of 13 of 22 (59%) of patients with short segments of Barrett's. With more and more evidence suggesting that LARS may affect the natural history of Barrett's esophagus, and ultimately the risk of esophageal cancer, surgical therapy should be strongly considered for patients with Barrett' s esophagus, especially for young patients and those with symptomatic Barrett's esophagus.

Respiratory symptoms

GERD has long been associated with respiratory symptoms (chronic cough, sinusitis, and recurrent pneumonia), asthma and laryngeal injury (hoarseness, subglottic stenosis, and laryngeal cancer)[16]. A leading theory suggests that decreased esophageal peristalsis and delayed acid clearance leads to persistent aspiration and subsequently to injury within the respiratory tract. Respiratory complaints in patients with GERD are synonymously referred to as atypical symptoms of GERD, laryngopharyngeal reflux (LPR), or laryngoesophageal reflux disease (LERD). And even though atypical GERD symptoms are much more likely to respond to surgical therapy than medical therapy, the most useful predictor for the relief of reflux-related respiratory symptoms through LARS is a prior positive response to PPI therapy[17].

Johnson *et al*[18] found that patients with typical symptoms of GERD frequently have respiratory symptoms. In their series of patients undergoing Nissen fundoplication for typical symptoms of GERD, 76% of patients who had respiratory symptoms preoperatively, experienced relief of their symptoms postoperatively. Similarly, Farrell *et al*[19] reported on 324 patients who underwent laparoscopic fundoplication for typical and atypical symptoms of GERD. They found that 99% of patients with predominantly typical symptoms experienced symptom relief, whereas 93% of those with primarily atypical symptoms (asthma,

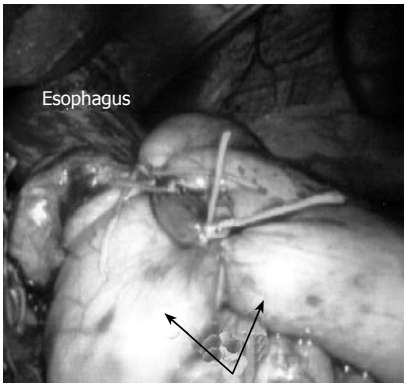


Figure 1 The Nissen fundoplication involves a 360-degree wrap (arrows) of the gastric fundus around the distal esophagus.

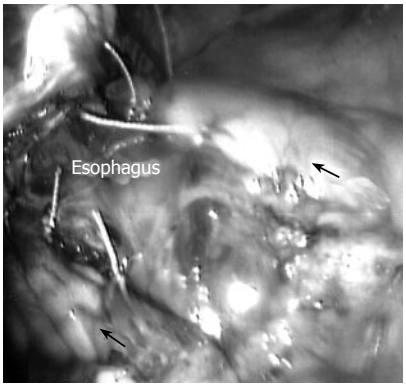


Figure 2 With the Toupet fundoplication, the gastric fundus (arrows) is incompletely wrapped around the posterior, distal esophagus.

cough, and hoarseness) experienced relief. Recently, it has become increasingly evident that GERD can be associated with non-allergic asthma. Spivak *et al*[20] reported significantly improved asthma symptoms for 39 patients who underwent antireflux surgery for the primary indication of asthma associated with GERD.

Total vs partial fundoplication

The total (Nissen) fundoplication involves a 360-degree wrap of the gastric fundus around the esophagus (Figure 1) and is the most commonly performed anti-reflux operation. Further insight into esophageal motility disorders and concerns of postoperative dysphagia has prompted the discussion whether the total or partial fundoplication is more appropriate treatment for GERD. Total wrap supporters acknowledge the fact that the wrap needs to be "floppy" to minimize postoperative dysphagia. It should also be noted that a floppy Nissen fundoplication is safe and effective in patients suffering from a defective esophageal peristalsis. Finally, proponents of the total fundoplication note the decreased the effectiveness of a partial fundoplication in controlling reflux.

Two partial fundoplications are in common practice; they are the Dor and Toupet fundoplications. Of these two partial wraps, the Toupet is the most commonly performed partial fundoplication (Figure 2). Fibbe *et al*[21] compared laparoscopic partial and total fundoplications in 200 patients with esophageal motility disorders and found no difference in postoperative recurrence of reflux and concluded that no tailoring of the surgical management is necessary. Similarly, Laws *et al*[22] found no clear advantage of one wrap over the other in their prospective, randomized study comparing these two groups. Moreover, in a meta-analysis of 9 prospective randomized trials including open and laparoscopic total *vs* partial fundoplications in 793 patients, no statistical difference was found in new-onset dysphagia or recurrence of reflux[23].

Dividing the short gastric vessels

The effect of fundic mobilization has been discussed widely in the surgical community. A meta-analysis of 4 randomized controlled trials found that the operative time is slightly, but not significantly longer when the superior-most short gastric vessels are divided[23]. No significant differences were found regarding the incidence of postoperative dysphagia or complications by dividing the short gastric vessels. The authors of this review favor the complete mobilization of the gastric fundus with division of the short gastric vessels because it is usually necessary for the creation of a "floppy" fundoplication.

Endoluminal therapy

Recent interest in the development in endoluminal therapy has led to new alternatives in the treatment of GERD. These treatment modalities are primarily designed for patients with little or no significant hiatal hernia. These therapies are delivered trans-orally, via upper endoscopy. There are principally three different types of these new treatment options. First, radiofrequency energy delivered to the lower esophageal sphincter (LES) theoretically adds bulk to the LES and changes the sphincter's compliance. Although the mechanism of action has not been fully established, it is presumably due to neurolysis and the abolishment of neuromodulated LES relaxation. With the Stretta Catheter (Curon Medical, Sunnyvale, CA, USA), radiofrequency energy is generated by a four-channel radiofrequency generator, which delivers 465 kHz/2-5 W per channel/80 V through 4 nickel-titanium needle electrodes. The energy is distributed to the LES submucosal/muscular target area at temperatures less than 100 ℃. Multiple prospective non-randomized studies with short-term results, up to 12 mo, showed promising results. Triadafilopoulos *et al*[24] showed improved quality of life scores, decreased esophageal acid exposure, decreased median DeMeester scores (40.0 to 26.4, $P < 0.009$) and 70% of patients off PPI at 12 mo for a group of 118 patients. Additional data on long term follow up, including the incidence and natural history of esophagitis in these patients, are required to establish the utility of this procedure.

A second endoscopic therapy for GERD involves the creation of a mechanical barrier at the gastroesophageal junction (GEJ). Enteryx (Boston Scientific, Natick, Massachusetts) is an ethylene vinyl alcohol copolymer that is endoscopically injected within the submucosa or muscular layers of the esophageal wall 1-2 mm caudal to the Z line. This technique has recently been discontinued by the company due to excessive complications[25]. The Gatekeeper Relux Repair System (Medtronic, Tolochenaz, Switzerland) entails the submucosal placement of a poly-acrylonitrile-based hydrogel prosthesis (1.5 mm × 18 mm) above the GEJ. In a prospective, nonrandomized study of 68 patients with a 6 mo follow up, quality of life was improved,

median LES pressure (8.8 to 13.8 mmHg, $P < 0.01$) was increased and 70.4% of the prostheses were still in place at 6 mo[26]. Concerns remain about the durability of the implants, leaving the long term utility of the procedure in question.

The third category of endoluminal procedures employs direct, endoscopic tightening of the LES. This can be achieved by either sewing or plicating the LES. Currently, three endoscopic suturing devices are available: the EndoCinch (BARD Endoscopic Technologies, Billerica, Massachusetts, USA), the ESD (Wilson-Cook Medical, Winston-Salem, North Carolina, USA), and the Full-Thickness Plicator (NDO Surgical Inc., Mansfield, Massachusetts, USA). The major difference is that the NDO Plicator takes a full thickness suture of gastric fundus whereas the other endoscopic plicators utilize a partial mucosal/submucosal stitch. Partial-thickness sutures have a greater potential to pull through the tissue or migrate over time. The NDO Plicator system appears to have the technical advantages of a single full thickness stitch, with serosa to serosa approximation, leading to direct tightening of the LES, possible lengthening of the LES and favorable altering of the angle of His. Pleskow *et al*[27] showed in a non-randomized, prospective study of 64 patients with follow-up of 12 mo that mental and physical SF-36 scores were significantly improved. Importantly, 80% of patients had improved distal esophageal acid exposure as evidenced by significantly decreased DeMeester scores (44 to 30, $P < 0.001$). Moreover, the majority of patients (68%) were off PPI at 12 mo follow-up.

The NDO Plicator as well as the other endoluminal therapies need to be evaluated very carefully in randomized studies. They will probably not replace the existing LARS but possibly serve as a "bridging therapy" finding its place between the Noninvasive GERD therapy and LARS.

ACHALASIA

Heller[28] was the first surgeon to perform an esophagomyotomy for achalasia in Germany in 1913. Over the next several decades, most of the discussion about myotomy centered on the preferred approach to myotomy, whether via an abdominal incision or using a thoracic technique. This discussion became moot in the 1990s as both approaches were supplanted by the laparascopic technique. The first laparoscopic esophagogastromyotomy was performed by Shimi *et al*[29] in 1991. Current controversies in the treatment of achalasia include the allocation of appropriate therapy, i.e. medical treatment, pneumatic dilation, or surgical treatment, to patients with achalasia. All of these treatments are designed to alleviate the symptoms of achalasia by addressing the failure of the LES to relax upon swallowing. However, in none of these therapies the underlying aperistalsis of the esophagus is addressed.

Pharmacotherapy

Medications are the only true noninvasive option in the treatment of achalasia. However, the medications used to treat achalasia have a short half-life and are variably absorbed due to the poor esophageal emptying experienced by patients with achalasia. As the disease progresses, this treatment modality becomes less and less effective. Calcium channel blockers and nitrates have been widely used, with the sublingual application being preferred[30,31]. Both medications effectively reduce the baseline lower esophageal sphincter tone, but they neither improve LES relaxation nor augment esophageal peristalsis. Persistent dysphagia and adverse effects including headaches, hypotension and peripheral edema make this treatment even more problematic[30,32]. The current recommendations for pharmacotherapy are limited to patients who are early in their disease process, without significant esophageal dilatation, or patients who are high risk for more invasive modalities. Finally, medications should be considered for patients who decline other treatment options.

Botulinum toxin

Direct injection into the lower esophageal sphincter with Botulinum toxin A (Botox, Allergan, Irvine, California) irreversibly inhibits acetylcholine release from cholinergic nerves. This results in decreased constriction of the sphincter and a temporary amelioration of achalasia related symptoms. Following Botox injection, about 60%-85% patients suffering from achalasia will note an improvement in their symptoms[33]. However, in Botox responders, symptoms recur in more than 50% of patients by 6 mo[34,35]. While repeated injections are technically possible, reduced efficacy is seen with each subsequent injection. Repeated esophageal instrumentation with Botox treatment inevitably causes an inflammatory reaction at the gastroesophageal junction. The resulting fibrosis may make subsequent surgical treatment more difficult and/or increase the complication rate. Fortunately, there has been recent evidence that despite Botox therapy, laparoscopic Heller myotomy is as successful as myotomy in Botox-naïve patients. However, prior Botox injection did increase the intraoperative esophageal perforation rate from 2% to 7%[36]. Botulinum toxin therapy is generally recommended for patients who are unwilling to undergo a more invasive treatment.

Pneumatic dilation

Since the original 17th century description by Willis[37], who used a sponge attached to the distal end of a whale bone to dilate a distal esophageal obstruction, the idea of stretching the lower esophagus (today with polyethylene balloons) is still considered the most effective nonsurgical treatment for achalasia. Under direct endoscopic visualization, a balloon is placed across the LES and is inflated up to 300 mmHg (10-12 psi)for 1-3 min. The goal is to produce a controlled tear of the LES muscle to render it incompetent, thereby relieving the obstruction. After pneumatic dilation, good to excellent response rates can be achieved in 60% of patients at one year[38]. Long-term results are less satisfactory. At 5 years, only 40% note symptom relief and at 10-year follow-up, a mere 36% of patients have relief of dysphagia[39,40]. Repeat dilatations are technically possible but show a decreased success rate. The most feared complication of pneumatic dilatation is a perforation of the distal esophagus, which frequently occurs at the left posterior lateral aspect (Boerhaave.s triangle). Risk factors for perforation include a widely dilated

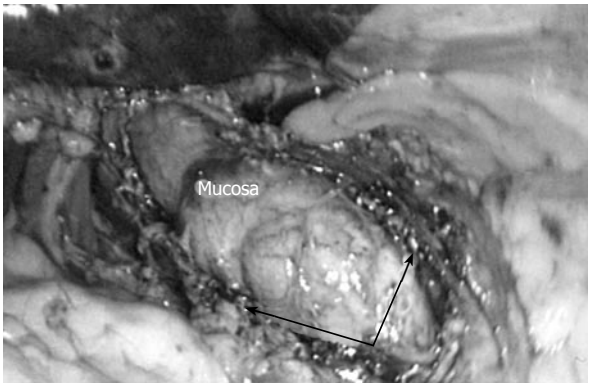


Figure 3 The goal of the Heller myotomy is full-thickness division of the muscular layers (arrows) of the distal esophagus. The myotomy should be long enough to ensure complete obliteration of the lower esophageal sphincter.

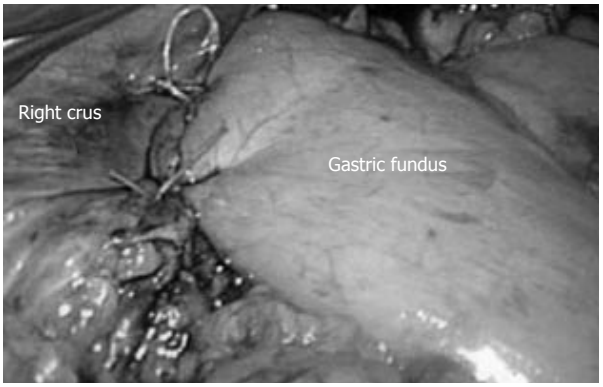


Figure 4 The Dor fundoplication is a 180-degree *anterior* folding of the gastric fundus over the distal esophagus.

esophagus (>7 cm), hiatal hernia, or the presence of an epiphrenic diverticulum. The rate of esophageal perforations during pneumatic dilation is approximately 2%[41]. The current recommendations regarding pneumatic dilation prior to (or instead of) surgical therapy depends upon the referring physician's opinions, the surgeon's expertise, and the patient's preference.

Surgical therapy

Heller first described the surgical destruction of the gastroesophageal sphincter, as therapy for achalasia, in 1913. His original technique used 2 parallel myotomies that extended for at least 8 cm on the distal esophagus and proximal stomach. The conversion to a single anterior myotomy was first proposed by De Brune Groenveldt in 1918. During the 1950s, the thoracic approach to esophagomyotomy was perfected, and in parts of the world, became the preferred technique. Despite the excellent results in improving the dysphagia, open Heller myotomy had a prolonged hospital stay and recovery time. Since the inception of minimally invasive techniques, both the open transabdominal repair and the thoracic approach have fallen out of favor.

First performed by Pellegrini *et al*[42] in 1992, the thoracoscopic approach showed promise, with excellent symptom relief. Unfortunately, technical factors limit the extension of the myotomy well onto the cardia of the stomach and it also makes a concomitant antireflux procedure difficult. Consequently, a higher rate of persistent and recurrent dysphagia (up to 26%) and GERD (35%) occur with this approach. When compared to the laparoscopic technique, this approach results in more postoperative pain and a longer hospital stay.

At this time, the laparoscopic approach is considered superior to the above mentioned surgical therapies. Laparoscopic Heller myotomy (Figure 3) is noted to have a very low morbidity and mortality rate, less postoperative pain, improved cosmesis, and a faster recovery compared to more invasive surgical therapies. Most importantly, excellent symptom relief is noted in 90% of patients[43]. This has established the laparoscopic approach as the premier choice for the treatment of achalasia.

The current indications for laparoscopic surgical treatment of achalasia include patients less than 40 years of age, patients with persistent or recurrent dysphagia after unsuccessful Botox injection or pneumatic dilation, and patients who are high risk for perforation from dilation. This latter group includes patients who have previously undergone surgery at the GE junction, those with esophageal diverticula, and those with distorted lower esophageal anatomy. Patients may prefer surgical therapy for a more durable, long-term result, its superior safety profile, and the decreased likelihood of reintervention.

Currently there are two controversies concerning achalasia discussed within the surgical community. The first involves the extent to which the myotomy is extended onto the stomach. Everyone concurs that a surgical myotomy predisposes patients to postoperative GERD; many surgeons also add an antireflux procedure to address this potential problem. Thus the second controversy involves whether to add an antireflux procedure, and if so, which type of antireflux procedure.

The proximal extent of the myotomy is typically carried 5-6 cm proximal to the lower esophageal sphincter. Whether to carry the distal extent of the traditional 1-2 cm beyond the LES or to be more aggressive and carry the myotomy at least 3 cm onto the stomach is now debated. Oelschlager *et al*[44] found less dysphagia and subsequently fewer interventions to treat the recurrent dysphagia in favor of the at least 3 cm distal myotomy group (3% *vs* 17%).

The necessity and type of antireflux procedure to prevent GERD after myotomy is the second widely discussed topic. Arguments against performing any antireflux procedure include some surgeo's reports of an adequate cardiomyotomy without postoperative reflux. Additionally, performing a fundoplication will obviously prolong the operative time, and there is also a proposed concern about adding a fundoplication in the setting of an aperistaltic esophagus. Proponents supporting the addition of an antireflux procedure mostly favor a partial (Dor, Toupet) over a total (Nissen) fundoplication to avoid a functional obstruction (from the fundoplication) and the persistence of dysphagia.

The anterior, 180-degree, Dor fundoplication (Figure 4) and the posterior, 270-degree Toupet fundoplication are the two most commonly performed antireflux procedures

in combination with the Heller myotomy. Unfortunately, the lack of prospective randomized studies comparing these two procedures leads to no clear advantages of one partial fundoplication over the other. Proponents of the posterior fundoplication believe that it better prevents re-approximation of the myotomy and believe that it is more effective against GERD. Advocates of the anterior fundoplication procedure argue that it is easy to perform, it protects the anterior esophagus following myotomy and it leaves the posterior anatomy intact. Acknowledging these facts, the authors believe the anterior fundoplication is the best technique to alleviate dysphagia and control reflux symptoms.

In conclusion, LARS and laparoscopic Heller myotomy are the agreed upon gold standard for surgical treatment of GERD and achalasia, respectively. Both are safe and effective treatment for patients with lower morbidity and mortality, shorter hospital stay and recovery period and less postoperative pain when compared to traditional open procedures. Additionally, excellent subjective and objective long-term results with at least 90% patient satisfaction have been observed, making LARS and laparoscopic Heller myotomy the treatment of choice in the experienced hands of dedicated minimally invasive surgeons.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Choledocholithiasis: Evolving standards for diagnosis and management

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Abstract

Cholelithiasis, one of the most common medical conditions leading to surgical intervention, affects approximately 10 % of the adult population in the United States. Choledocholithiasis develops in about 10%-20% of patients with gallbladder stones and the literature suggests that at least 3%-10% of patients undergoing cholecystectomy will have common bile duct (CBD) stones.

CBD stones may be discovered preoperatively, intraoperatively or postoperatively Multiple modalities are available for assessing patients for choledocholithiasis including laboratory tests, ultrasound, computed tomography scans (CT), and magnetic resonance cholangiopancreatography (MRCP). Intraoperative cholangiography during cholecystectomy can be used routinely or selectively to diagnose CBD stones.

The most common intervention for CBD stones is ERCP. Other commonly used interventions include intraoperative bile duct exploration, either laparoscopic or open. Percutaneous, transhepatic stone removal other novel techniques of biliary clearance have been devised. The availability of equipment and skilled practitioners who are facile with these techniques varies among institutions. The timing of the intervention is often dictated by the clinical situation.

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Key words: Choledocholithiasis; Laparoscopy; Diagnosis; Treatment; Cholangiogram

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INTRODUCTION

Symptomatic gallstone disease is a very common indication for abdominal surgery. It is estimated that around 500 000 cholecystectomies are performed a year in the United States. Gallstones, alone, are rarely an indication for surgery, but 10% of the adult population are believed to carry them. Furthermore, up to one-third of the population over 70 years of age will have gallstones. Gallstone formation is a multifactorial process but is undoubtedly associated with family history, diabetes mellitus, pregnancy, obesity, significant weight loss, and hemolytic diseases. As many as 35% of patients with gallstones will ultimately become symptomatic and require cholecystectomy[1].

Common indications for surgical treatment for cholelithiasis include biliary colic, acute cholecystitis, gallstone pancreatitis, and other presentations of choledocholithiasis including bile duct obstruction and cholangitis. Other relative indications include gallstones in patients undergoing splenectomy for hemolytic anemia, high risk patients in the pretreatment phase for other conditions such as bone marrow transplant. Cholecystectomy is no longer routinely performed for asymptomatic gallstones in those undergoing bariatric surgery or aortic surgery.

Common bile duct (CBD) stones may be discovered preoperatively, intraoperatively or postoperatively. The standard preoperative workup for patients presenting with symptoms attributable to cholelithiasis includes liver function tests, and abdominal ultrasound. These tests, combined with clinical exam and history, constitute the entire workup for most patients. Abnormalities in these tests may suggest the presence of choledocholithiasis. Choledocholithiasis may occur in up to 3%-10% of all cholecystectomy patients[1], or as high as 14.7% in some series[2]. This includes some patients without classic preoperative findings suggestive of choledocholithiasis. Of these asymptomatic patients, it is believed about 15% will eventually become symptomatic[3] and require further interventional treatment.

Since the advent of routine laparoscopic cholecystectomy, the debate has continued about the utility of intraoperative bile duct assessment, primarily with cholangiography or intraoperative ultrasonography. This debate continues to some degree, with most surgeons either selectively or routinely evaluating the bile duct intraoperatively. A smaller subset relies only on preoperative assessment tools, including magnetic resonance cholangiopancreatography (MRCP)

and endoscopic retrograde cholangiopancreatography (ERCP), as a complement to routine laboratory and imaging studies. The most common intervention for common bile duct (CBD) stones is ERCP. Other procedures for CBD stone extraction include percutaneous, transhepatic stone removal and intraoperative CBD exploration, whether laparoscopic or open. The availability of equipment and skilled practitioners who are facile with these techniques varies among institutions. The timing of the intervention is often dictated by the clinical situation.

PREOPERATIVE EVALUATION AND THERAPY

Diagnosis of choledocholithiasis is not always straightforward and clinical evaluation and biochemical tests are often not sufficiently accurate to establish a firm diagnosis. Imaging tests, particularly abdominal ultrasound, are used routinely to confirm the diagnosis. Liver function tests (LFT) can be used to predict CBD stones[4,5]. Elevated serum bilirubin and alkaline phosphatase typically reflect biliary obstruction but these are neither highly sensitive nor specific for CBD stones. Excepting obvious jaundice, a raised GGT level has been suggested to be the most sensitive and specific indicator of CBD stones. A value of greater than 90 U/L has been proposed to indicate a high risk of choledocholithiasis[4]. However, laboratory data may be normal in as many as a third of patients with choledocholithiasis, warranting further evaluation of the CBD by imaging studies to clarify the diagnosis.

In order to help select from the various diagnostic and therapeutic options, patients may be classified preoperatively into high, moderate or low risk groups. The high risk (> 50% risk) group includes those patients with obvious clinical jaundice or cholangitis, choledocholithiasis or a dilated CBD on ultrasonography. Patients with a history of pancreatitis or jaundice, elevated preoperative bilirubin and alkaline phosphatase levels or multiple small gallstones carry a moderate (10%-50%) risk of choledocholithiasis. Patients with large gallstones, without a history of jaundice or pancreatitis and with normal liver function tests are considered unlikely to have CBD stones and therefore at low risk (< 5%)[6-8].

The transabdominal ultrasound examination (US) is the most commonly used screening modality. In patients who present with symptoms attributable to gallstone disease, US may be the only radiologic study ordered. It has the advantages of being widely available, non-invasive, and inexpensive. Ultrasonography, however, is highly operator dependent, but it can provide useful information in experienced hands. For a diagnosis of cholecystitis, in most circumstances, gallbladder stones need to be visualized. The sonographic presence of wall thickening, pericholecystic fluid, and a Murphy's sign, may be helpful but are not required for a diagnosis of acute cholecystitis. The ultrasonographer should routinely report indirect information suggestive of the presence or absence of CBD stones, specifically the CBD diameter or any signs of intrahepatic bile duct dilation. The sensitivity of US for detecting biliary dilatation, as reported in various studies, varies from 55 to 91 percent[9]. A CBD diameter of greater than 6 mm on US is associated with a higher prevalence of choledocholithiasis[10].

Computed tomography (CT) may have some role in diagnosis of gallstone disease and choledocholithiasis. Many patients presenting with acute abdominal pain will undergo a diagnostic CT scan as part of the acute workup. A diagnosis of acute cholecystitis may be evident based on signs of gallbladder inflammation. Cholelithiasis may be detected on CT[11] and often the diameter of the CBD can be measured. In the clinical setting, US may not be necessary for preoperative evaluation if the CT scan provides this information. The role of helical CT cholangiography is still in evolution, particularly in the United States. Intravenously administered contrast agents, combined with high resolution helical scans and three dimensional reconstructions that can be very useful in diagnosing choledocholithiasis[12,13]. The sensitivity of this technique can be as high as 95.5%[12]. This technique is not widely utilized in the U.S. as the available contrast agents often cause significant nausea on administration. The availability of MRCP also limits the need for this modality.

MRCP has emerged as an accurate, non-invasive diagnostic modality for investigating the biliary and pancreatic ducts[14] and has been recommended in some circles as the preoperative procedure of choice for the detection of CBD stones[4,15,16]. MRCP provides excellent anatomic detail of the biliary tract and has a sensitivity of 81%-100% and a specificity of 92%-100% in detecting choledocholithiasis[14]. The accuracy of MRCP in diagnosing CBD stones is comparable with that of ERCP and IOC[14,17]. It thus avoids the need for a potentially high risk, invasive procedure in more than 50% of patients, allowing selective use of ERCP or surgical CBD exploration in those patients who require a therapeutic intervention. These results have led some practitioners to consider MRCP the new gold standard for biliary imaging[14,18]. MRCP may, however, miss stones less than 5 mm in diameter. MRCP is an expensive option that requires significant expertise for interpretation; this modality may not always be readily available.

Endoscopic techniques such as endoscopic ultrasound (EUS) and ERCP can also be useful tools in preoperative diagnosis and, in the case of ERCP, management of choledocholithiasis. Both procedures are more invasive than strictly radiologic techniques, and carry the low, but inherent risks of upper gastrointestinal tract endoscopy. ERCP also carries the risks associated with biliary instrumentation, such as pancreatitis. Endoscopic ultrasound (EUS) can been used to evaluate the CBD and identify calculi. Studies comparing the accuracy of EUS to US, CT and ERCP for detecting choledocholithiasis show a sensitivity of EUS ranging from 88%-97%, with a specificity of 96%-100%[19]. This is comparable to ERCP and avoids the risks of pancreatitis, cholangitis and radiation exposure[19]. The role of EUS, however, is not well established especially when cost and availability of less invasive modalities such as MRCP are considered. EUS may be combined with ERCP at the same endoscopy session if biliary calculi are identified, allowing it to be a bridge to therapeutic intervention.

ERCP has been the gold standard for preoperative diagnosis of CBD calculi. When compared to other tests such

as ultrasonography and MRCP, ERCP has the advantage of providing a therapeutic option when a CBD stone is identified. Stone retrieval and sphincterotomy has supplanted surgical treatment of choledocholithiasis in many institutions[14,20]. Successful cholangiography by an experienced endoscopist is achieved in greater than 90% of patients. Complications associated with ERCP can be as high as 15% and include pancreatitis, cholangitis, perforation of the duodenum or bile duct, and bleeding. These individual complications can occur in 5%-8% of patients. The mortality rate from ERCP is 0.2%-0.5%[21,22]

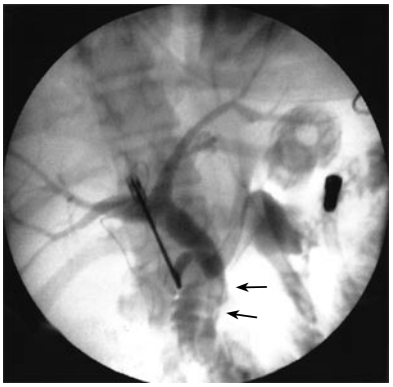


Figure 1 Intraoperative cholangiogram *via* the cystic duct demonstrating proximal biliary dilation and two filling defects in the CBD (Arrows).

INTRAOPERATIVE EVALUATION AND THERAPY

Since laparoscopic cholecystectomy became a routine procedure in the early 1990's, debate has continued about the role of intraoperative bile duct assessment. The interest in intraoperative cholangiography (IOC) came from both the desire to detect CBD stones and to potentially identify any bile duct abnormalities, including iatrogenic injuries at the time of surgery. Routine cholangiography is not generally considered to be the standard of care but still has its proponents[3]. Most surgeons selectively evaluate the bile duct intraoperatively. A smaller subset relies only on preoperative assessment tools including magnetic resonance cholangiopancreatography (MRCP) and endoscopic retrograde cholangiopancreatography (ERCP) as a complement to routine laboratory and imaging studies.

Intraoperative cholangiography can be a useful tool to identify choledochal calculi but its application remains controversial. Proponents often believe that IOC enables clear definition of the biliary tree anatomy[3,23,24], thus reducing the risk of bile duct injury during cholecystectomy. Most studies on the subject show no significant difference in the rates of ductal injury between routine and selective IOC. Routine IOC has been found to yield little benefit over the selective approach in the detection of symptomatic CBD stones[3] Cholangiography is a relatively straight forward procedure to perform. It does add to the operative time, approximately an additional 15 min[25,26], but surgeon and operative team experience can minimize this. Dynamic fluoroscopic imaging is the technique of choice and can provide a specificity and sensitivity of 94% and 98%, respectively, in experienced hands[27]. This technique may be somewhat less sensitive for patients presenting with biliary pancreatitis[27]. A complete IOC should demonstrate the cannulation of the cystic duct, filling of the left and right hepatic ducts, CBD and common hepatic duct diameter, the presence or absence of filling defects in the biliary tree, and free flow of contrast into the duodenum (Figure 1). Obstruction or other biliary abnormality should be suspected if these findings are not clear.

The overall safety profile of IOC is very good. The incidence of pancreatitis, in contrast to ERCP, is negligible[28]. The reliability, ease to perform, and low complication rate of IOC suggest that, at least in cases where choledocholithiasis is not proven, IOC during a cholecystectomy is a better choice than preoperative ERCP assessment of the bile ducts. More recently intraoperative ultrasonography of the CBD during laparoscopic cholecystectomy has been shown to satisfactorily demonstrate stones and the biliary tree. Use of intraoperative ultrasound requires a significant learning curve, but in experienced hands takes less operative time than traditional IOC[25,26]. Laparoscopic ultrasound compares well with intraoperative cholangiography. With a sensitivity of 96% and a specificity of 100%, and the advantages of saved time and lack of radiation, intraoperative ultrasound may be a superior diagnostic modality when compared to IOC[23]. The use of intraoperative laparoscopic ultrasound is limited by the availability of equipment and surgeon training and experience. Efforts to incorporate ultrasonography training in to surgical residency curricula should aid its acceptance and use.

When CBD stones are detected intraoperatively, a surgeon must make a clinical decision on how to proceed. This decision is often dictated by the availability of equipment, surgeon preference and skill, and the availability of ERCP at a facility. Available options include laparoscopic CBD exploration, intraoperative ERCP, open CBD exploration, or postoperative therapy. Many surgeons are reluctant to proceed with an open procedure when other, less invasive, options are generally available. However, a decision to delay therapy to the postoperative period risks encountering procedural difficulties that preclude endoscopic therapy. Options for intraoperative CBD clearance include variations of laparoscopic exploration. Laparoscopic CBD exploration is most commonly performed with acholedochoscope. The scope, attached to a second video camera and light source, is inserted into the CBD *via* either the cystic duct (Figure 2) or *via* a choledochotomy. Placement of the choledochoscope directly into the CBD *via* a choledochotomy is generally reserved for patients with ducts dilated to greater than 6 mm in diameter on cholangiogram[20]. A continuous saline infusion through the choledochoscope dilates the duct and clears debris. CBD stones can be directly visualized (Figure 3) and instrumented. Between 66 and 82.5% of laparoscopic CBD explorations can be performed via the cystic duct[20,29]. In experienced hands, the CBD clearance rate is as high as 97%[2,29]. In one large series of laparoscopic CBD exploration, the overall morbidity rate of this procedure is approximately 9.5%, with a 2.7% retained stone rate[2]. These data are equivalent to the experience with ERCP.

An alternate technique of laparoscopic CBD clearance involves stone extraction under fluoroscopic guidance.

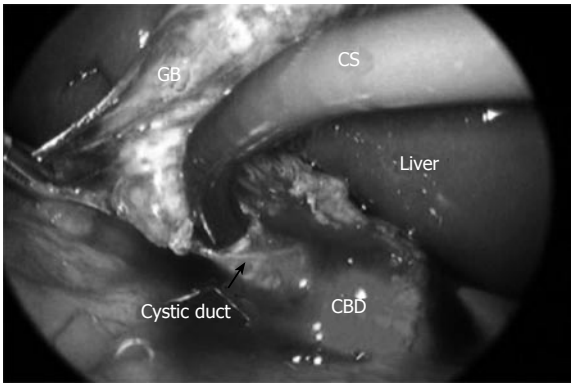


Figure 2 Laparoscopic view of a choledochoscope (CS) entering the CBD *via* the cystic duct. The gallbladder (GB) is retracted to the left of the image.

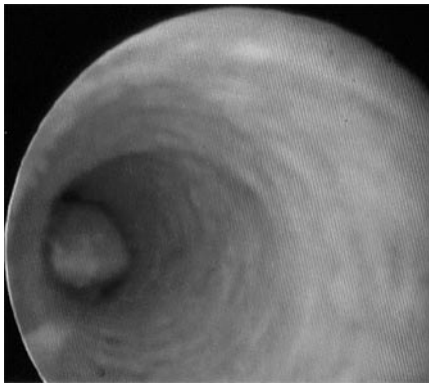


Figure 3 CBD stone as seen through the choledochoscope.

This technique, as described by Traverso *et al*, does not use a choledochoscope, but can be highly effective in CBD clearance for single stones (87%), with an overall 59% success rate[30]. In this technique, instruments, including stone extraction baskets, are passed into the CBD (usually via the cystic duct) under fluoroscopy. The fluoroscopic images facilitate stone capture and removal. This technique simplifies the operating room set up for CBD exploration because additional video equipment is not required. In this series, patients whose ducts were not cleared laparoscopically, underwent ERCP. The authors noted a significantly lower complication rate in patient who did not undergo ERCP[30]. Likewise, the overall associated costs were lower in these patients[31].

Laparoscopic techniques can be very effective at clearing CBD stones, but significant expertise and experience is required to achieve high success rates. Stones impacted at the sphincter of Oddi are often the most difficult to extract by this technique. Long term follow up does not demonstrate a significant risk of CBD stricture or other complications for these procedures[2,29,30]. This technique is also advocated in the pediatric population by some practitioners as a way of avoiding an ERCP[32]. Intraoperative ERCP, although often limited by the immediate availability of a qualified endoscopist, is a technique that can be very useful. This takes advantage of the preexisting anesthetic and does not prolong hospital stay by delaying the procedure[33,34]. In some instances, cooperation between the surgeon and the endoscopist can expedite the procedure[35]. Guidewire placement, throught the sphincter of Oddi, into the duodenum via the IOC catheter can facilitate cannulation, especially for patients with difficult duodenal anatomy[34]. In the case of an unsuccessful extraction, open CBD exploration or drainage of the duct should be considered.

POSTOPERATIVE EVALUATION AND THERAPY

Postoperative ERCP is commonly used modality for CBD clearance when a stone is detected intraoperatively. This technique is highly effective[20], but can be complicated when anatomic variables such as duodenal diverticulae or unusual biliary anatomy, are encountered[36]. On the occasion that ERCP fails, a primary option includes returning to the operating room for a laparoscopic or open exploration. However, other less invasive options may be available.

In selected patients, percutaneous transhepatic (PTH) therapies should be considered for CBD clearance. Access to the biliary system can be obtained percutaneously, under ultrasound guidance. Via this access point, stones can be extracted, a sphincterotomy can be performed, or lithotripsy can be used over a single, or multiple sessions to ensure duct clearance[37,38]. A percutaneous drain can be used to decompress the system between procedures or for temporary stenting after the duct is clear.

Novel approaches have been performed in other anatomic settings which preclude ERCP, such as prior gastric surgery. The most common gastric surgery currently performed is the Roux-Y gastric bypass. In this surgery, a small gastric pouch is created and anastomosed to a limb of jejunum. The majority of the stomach, duodenum, and proximal jejunum are bypassed, making endoscopic access technically difficult. Combined laparoscopic surgical and endoscopic procedures have been described. Endoscopic access can be achieved via a gastrostomy[39] or jejunostomy[40]. The endoscope can be passed under surgical guidance, and an ERCP can be performed in the standard fashion. These procedures are only described in short case reports, but will undoubtedly become more common place with the prevalence of gastric bypass.

In conclusion, given the various diagnostic and therapeutic options available for managing choledocholithiasis, clinical judgment is paramount. The surgeon and the patient are best served by accurate laboratory and radiologic assessment prior to cholecystectomy. In the setting of high preoperative suspicion for choledocholithiasis, preoperative imaging with MRCP may help guide subsequent treatment. If CBD stones are present on MRCP or in the setting of clinical jaundice or cholangitis, preoperative ERCP is the first line option. However, preexisting anatomic abnormalities may influence this decision. Laparoscopic IOC with a CBD exploration is a reliable approach in experienced hands and if an ERCP is unsuccessful.

Cholangiography during cholecystectomy is recommended for patients with a history of gallstone pancreatitis, LFT abnormalities, or an enlarged CBD on preoperative imaging who have not undergone MRCP or ERCP. IOC is also recommended when intraoperative findings suggest anatomic abnormalities or unsuspected CBD calculi.

Intraoperative laparoscopic CBD exploration is the first

option for choledocholithiasis detected in the operating room. Intraoperative or postoperative ERCP are options for incompletely cleared ducts. Percutaneous treatment or combined laparoendoscopic options are available in some centers for patients who cannot undergo ERCP or laparoscopic choledochotomy. Open CBD exploration is a safe option, but usually limited to the setting of concomitant open surgery or as a last resort when other therapeutic modalities fail.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Treatment strategies in obstructed defecation and fecal incontinence

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Abstract

Obstructed defecation (OD) and fecal incontinence (FI) are challenging clinical problems, which are commonly encountered in the practice of colorectal surgeons and gastroenterologists. These disorders socially and psychologically distress patients and greatly impair their quality of life. The underlying anatomical and pathophysiological changes are complex, often incompletely understood and cannot always be determined. As a consequence, many medical, surgical, and behavioral approaches have been described, with no panacea. Over the past decade, advances in an understanding of these disorders together with rational and similar methods of evaluation in anorectal physiology laboratories (ARP), radiology studies, and new surgical techniques have led to promising results. In this brief review, we discuss treatment strategies and recent updates on clinical and therapeutic aspects of obstructed defecation and fecal incontinence.

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Key words: Obstructed defecation; Fecal incontinence; Treatment

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INTRODUCTION

The prevalence of constipation in adults ranges from 2% to 27% in North America, particularly after the age of 65 and with a female predominance[1]. There are two major types of constipation: secondary constipation (due to colorectal cancer, hypothyroidism, hyperparathyroidism, diabetes, use of opiates, antidepressants, and diuretics, stool impaction in the elderly, sexual abuse in women, Parkinson's disease, and other organic pathology and medications) and primary functional constipation, which can be divided into slow transit constipation (colonic inertia), constipation-predominant irritable bowel syndrome, and obstructed defecation[2,3]. Approximately half of constipated patients suffer from obstructed defecation[4]. Obstructed defecation (OD)is a broad term of the pathophysiologic condition describing the inability to evacuate contents from the rectum. This disorder is commonly known by numerous terms, including paradoxical puborectalis contraction (PPC), outlet obstruction, anismus, pelvic floor dyssynergia, nonrelaxing puborectalis syndrome, spastic pelvic floor syndrome, and dyschezia. It may result from functional, metabolic, mechanical, and anatomical derangements involving a rectoanal evacuatory mechanism. Dyssynergia, or uncoordination of the pelvic floor muscles, leads to paradoxical external anal sphincter and puborectalis contraction with no relaxation during defecation[5].

OD may result from rectoanal intussusception, pelvic organ prolapse, rectocele, sigmoidocele, enterocele, solitary rectal ulcer syndrome, PPC and descending perineum syndrome. Other rare causes include rectal hyposensitivity (blunted rectum), idiopathic megarectum, hereditary internal sphincter myopathy and nutcracker anus[4,6].

This condition is usually defined on the basis of symptoms and physiologic and radiologic studies. Symptoms include a feeling of incomplete evacuation and rectal obstruction, passage of hard stools, rectal or vaginal digitation and excessive straining in the constipated patient (stool frequency of fewer than three times per week). Careful perineal and digital rectal examination, colonic transit time study, anorectal manometry, defecography or dynamic magnetic resonance imaging of the pelvic floor usually help assess defecatory dysfunction and establish a correct diagnosis[7,8].

Fecal incontinence (FI) is a second major problem with a devastating effect on patients' quality of life. Its prevalence ranges from 2% to 18% and increases with age especially in institutionalized and psychiatric patients and in women[9]. FI is usually attributable to multiple factors affecting the normal anatomy and physiology of anorectum. Obstetric trauma, anorectal surgery, benign

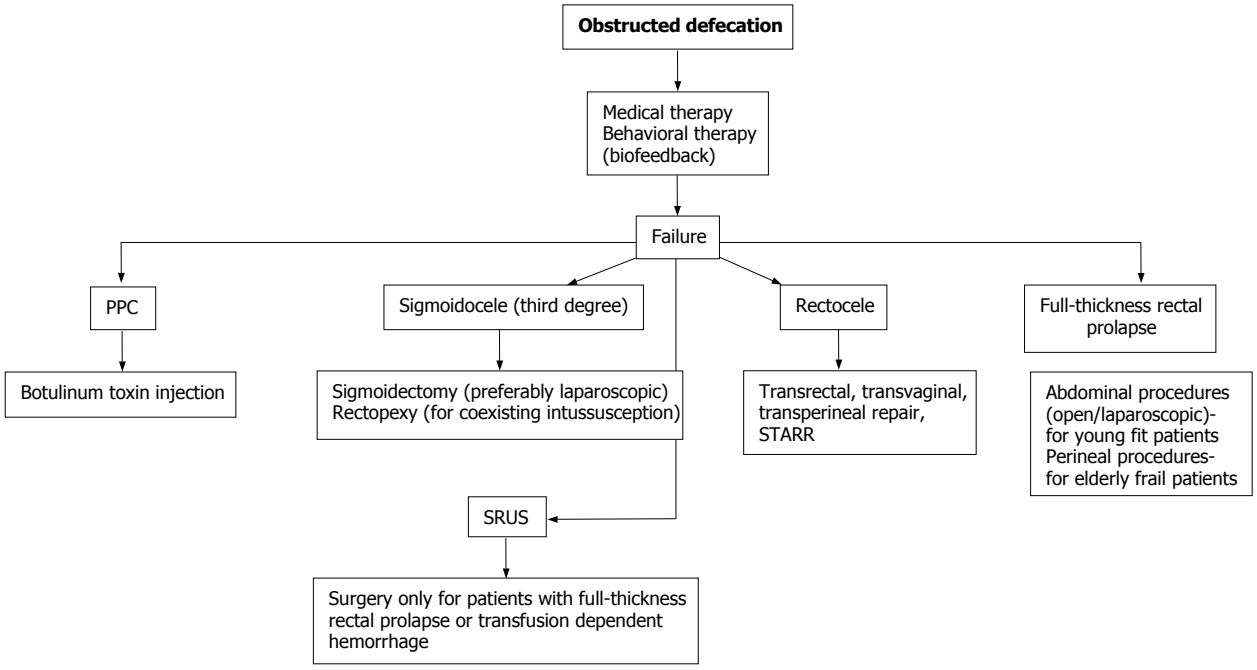


Figure 1 Treatment algorithm for obstructed defecation.

colorectal conditions, cancer, inflammatory bowel and neurologic diseases, aging, drugs, food intolerance, rectal prolapse, congenital abnormalities, and radiation proctitis are all known causes of FI. The most appropriate therapy for FI may be based only on an accurate assessment of anorectal function, including a thorough history, physical examination, anorectal physiology studies (endoanal ultrasound, anorectal manometry with balloon expulsion test, electromyography, and pudendal nerve latency testing) and other imaging tests (defecography, dynamic MR proctography)[10]. The main goal of treatment is to improve continence and the patients' quality of life.

TREATMENT STRATEGY

Obstructed defecation

Once secondary causes for constipation, especially malignancy and slow transit constipation (colonic inertia) are excluded, OD is considered on the basis of clinical examination, normal or prolonged colonic transit time, abnormal anorectal manometry, defecography, or dynamic MRI of the pelvic floor[11] (Figure 1).

Paradoxical puborectalis syndrome

Initial therapy for patients with PPC, as for descending perineum syndrome, solitary rectal ulcer, clinically non-significant rectocele, sigmoidocele, and rectoanal intussusception, is conservative. This includes a high fiber diet of up to 20-30 g/d, adequate hydration, regular physical activity, enemas, and laxatives.

Biofeedback training may be added to the first-line therapy, which teaches patients to relax their pelvic floor muscles[12-14]. There is a paucity of controlled trials showing the true effectiveness of this behavioral treatment. Furthermore, there is no single factor that can predict a favorable outcome with this treatment. In a large retrospective study from Cleveland Clinic Florida, Gilliland *et al* reported a 63% success rate of EMG-based biofeedback for constipation after five or more sessions[12]. They found that age, gender, duration of symptoms, and the coexistence of rectal pain did not influence outcome. In addition, there was no relationship between mean resting or squeeze pressure, length of the high pressure zone, sensory threshold, and maximum rectal capacity and success or failure of biofeedback. Chiarioni *et al* showed improvement in 71% of patients with OD for a period of two years[15]. Various biofeedback techniques (intra-anal and perianal EMG monitoring, manometric anal probe biofeedback, intra-rectal balloon expulsion biofeedback, ultrasound biofeedback) have been done, but none has a superior success rate, which ranges from 30% to over 90%[16-18]. Although the success rate seems to significantly wane with time, the morbidity-free nature of biofeedback makes re-treatment an attractive option.

Despite controversy about the method of biofeedback and the number of sessions needed, it seems reasonable to use this generally safe technique as the initial treatment for OD. After failure of conservative therapy, 50%-75% of patients with PPC may benefit from botulinum toxin type A injection into the puborectalis muscle and external anal sphincter. The short-term effect (3 mo), however, may require repeated injections[19,20]. Surgical division of the puborectalis muscle is usually non-effective and currently not a recommended procedure[21]. Several other treatments exist for this disorder, including progressive anal dilatation and sacral nerve stimulation (SNS). Additional studies are needed to completely evaluate these techniques for this indication.

Solitary rectal ulcer syndrome (SRUS)

This syndrome may be associated with PPC, rectoanal intussusception, rectal prolapse, and descending perineal syndrome. Chronic straining with ischemic ulceration

are components of the pathogenesis, which is still not completely clear. Biopsy from the lesion should be obtained to exclude malignancy. Treatment for this syndrome is conservative and is the same as for PPC, including high fiber and fluid intake, laxatives, suppositories, and biofeedback. Surgery is generally reserved for patients with full thickness rectal prolapse or intractable hemorrhage and should be tailored to the individual patient's operative risk, willingness, and continence and constipation status[22,23]. Regardless of which procedure is selected, it should be safe balancing anticipated morbidity with an acceptable recurrence rate. Unfortunately, a detailed description of the different surgical techniques is beyond the scope of this article.

Descending perineum syndrome

This syndrome is abnormal perineal descent possibly as a result of consistently prolonged straining with defecation, as defined on defecography. Medical therapy with a high fiber diet, laxatives, enemas, and biofeedback is the main treatment for these patients; no viable surgical option exists.

Rectocele

Treatment of rectocele is usually indicated when a herniation of the anterior rectal wall is greater than 3 cm with significant clinical symptoms or non-emptying rectocele on defecography. However, there is no correlation between the severity of symptoms, contrast material retention, the depth of a rectocele and success of the repair[24,25]. Rectocele presents on defecography in 81% of asymptomatic women[26]. The initial treatment is the same as for disorders previously outlined. Surgery may be considered when conservative therapy fails and includes transvaginal posterior colporrhaphy, and transrectal or transperineal repair. Careful patient selection is very important for a successful outcome. Two new techniques include the double stapled Trans-Anal Rectal Resection (STARR) and single stapled Trans-Anal Prolapsectomy with Perineal Levatorplasty (STAPL) for the management of OD associated with rectocele or intussusception. These methods use one or two 33-mm circular staplers (PPH-01 Ethicon-Endosurgery, Inc, Cincinnati, OH) to perform mucosal or full-thickness circumferential transanal resection of the distal rectum. In their randomized study Boccasanta et al reported significant symptomatic improvement after STARR and STAPL in 88% and 76%, respectively, at approximately 2 years[27]. These procedures are considered safe with minimal postoperative pain and high patient satisfaction[28].

Rectal intussusception (internal rectal prolapse)

Approximately half of the patients with rectal intussusception present with clinical symptoms of OD while in 29% it is seen on defecography in asymptomatic patients[29,30]. The mainstay of therapy is dietary management and biofeedback. Surgery is reserved only for patients with combined pathology, usually FI as a result of the external anal sphincter defects or pudendal neuropathy, or if the intussusception progresses to a full thickness rectal prolapse.

Sigmoidocele and enterocele

Deep herniation of the rectovaginal peritoneal cavity containing sigmoid colon or small bowel is usually associated with other manifestations of OD, rectocele, or pelvic organ prolapse. Sigmoidocele is confirmed by defecography. Jorge *et al* from the Cleveland Clinic Florida suggested a sigmoidocele classification based on the degree of descent of the lowest portion of the sigmoid colon[30]. First degree sigmoidocele is located below the sacral promontory but above the pubococcygeal line, second degree is below the pubococcygeal line and above the ischiococcygeal line, and third degree is below the ischiococcygeal line. Staging of sigmoidocele is useful in determining both clinical significance and optimal treatment.

Indications for surgery are symptomatic patients with a third-degree sigmoidocele (below ischiococcygeal line) or during other pelvic surgery, using abdominal or vaginal approaches (hysterectomy, rectal prolapse, rectocele repair). Surgery includes resection of the sigmoid colon (preferably laparoscopic sigmoidectomy), and rectopexy with obliteration of the Douglas pouch; some patients may benefit from dietary and biofeedback therapy. Jorge and colleagues reported symptomatic improvement in 100% of patients who underwent surgery, but in only 33% of patients who have been conservatively treated at a mean follow-up of 23 mo[30].

Fecal incontinence

Fecal incontinence is the result of numerous disorders affecting the anatomy and physiology of the anorectum. Many appropriate medical, behavioral, and surgical treatments exist to correct the underlying pathology and restore continence. Irritable bowel syndrome, inflammatory bowel disease, scleroderma (internal anal sphincter fibrosis), neurological (spinal cord injuries) and psychological problems (dementia, depression, anxiety), diabetes mellitus (autonomic neuropathy), skeletal muscle diseases (myopathy, myasthenia gravis), diarrhea, constipation, and fecal impaction should all be appropriately treated. However, when underlying pathology cannot be identified, the primary goal of the therapy is to relieve symptoms and improve patients' quality of life (Figure 2).

Initial therapy consists of dietary modifications to form stool, control diarrhea or constipation, and treat fecal impaction with overflow incontinence. This treatment includes perianal hygienic measures with barrier cream to prevent skin irritation and dermatitis, increased fiber and fluid uptake, avoidance of dairy products and caffeine, using stool softeners, osmotic laxatives (magnesium salts), and non-absorbable sugars (lactulose). Antidiarrheal agents (loperamide, diphenoxylate/atropine) increase fluid absorbtion, colonic transit time, and anal resting sphincter pressure[31]. Low dose amitriptyline (tricyclic antidepressant) may improve continence due to its anticholinergic and serotoninergic activities[32].

Biofeedback is another first-line therapy for FI. This method of operant conditioning, using visual verbal or auditory signals, improves rectal sensation, rectoanal coordination, and trains external anal sphincter

contractility. Success rates from various series range from 40% to 85%, most probably predicted by patient motivation rather than the duration of FI, manometric or endoanal ultrasound findings, or the biofeedback technique[33,34].

Several other invasive nonoperative techniques exist that may benefit patients with FI. The Procon incontinence device (Incontinence Control Devices, Inc., Kingwood, TX) is a soft catheter with a photosensor and balloon, which may be inserted into the rectum to send a signal warning of coming stool. This device has shown an improvement in the continence of select patients[35]. Submucosal injections of a wide variety of bulking agents have been used to augment the internal anal sphincter (silicon, silicone-based agent [Bioplastique ®], carbon-coated beads [Acyst™ procedure], carbon-coated zirconium oxide beads [Durasphere®], autologous fat, glutaraldehyde cross-linked collagen [GAX], polytetrafluoroethylene [Polytef]) have shown symptomatic improvement in anal resting pressure and incontinence. These office-based procedures are simple, relatively safe, and seemingly efficacious[36].

The Secca® procedure (Curon Medical, Inc., Sunnyvale, CA) uses temperature-controlled radiofrequency energy in the anal canal and distal rectum to create scarring of the internal anal sphincter and tissue fibrosis. In a prospective multicenter study, Efron et al reported improvement in FI and quality of life with resolution of symptoms in 60% of patients[37,38]. Takahashi et al reported similar findings at a two-year follow-up[39].

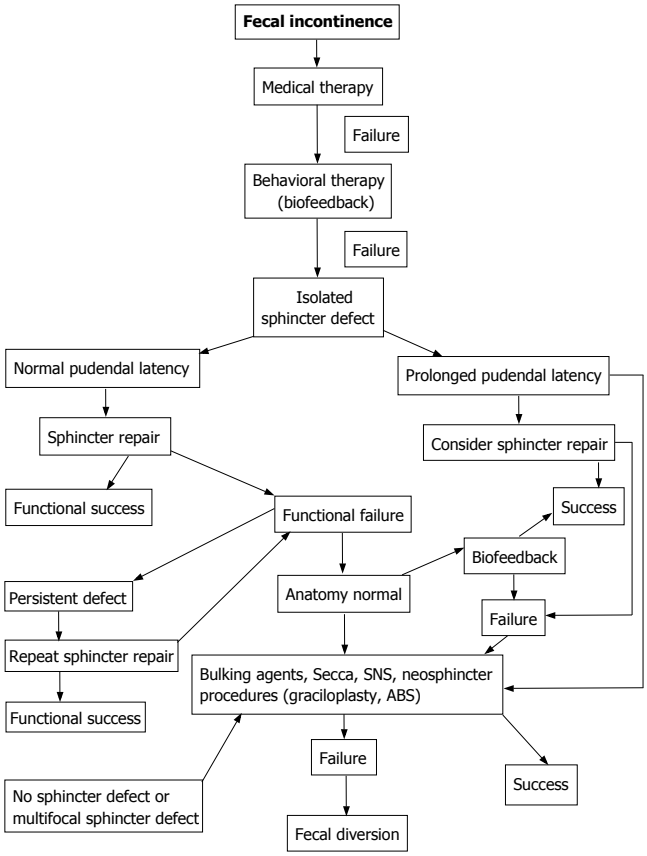


Figure 2 Treatment algorithm for fecal incontinence.

Surgery

Failure of conservative therapy in patients with external sphincter defects but without neurologic injury (intact pudendal nerves) are suited to surgical repair. Anterior defects from obstetric damage is the most common cause of FI in these cases. The surgical procedure is usually anterior overlapping sphincteroplasty, including preservation of all fibrotic scar and overlapping of both sides of the external anal sphincter with non-absorbable sutures[40]. Although approximately 80% of patients benefit from this operation in the short-term period, the rate of incontinence increases after 5-10 years by up to 85%[41-47].

Patients with severe incontinence with significant neurologic injury, multifocal external anal sphincter damage that cannot be primarily restored, or after functional failure of an anatomical successful sphincteroplasty, may be considered for reconstruction with neosphincter procedures using striated muscles (gracilis, gluteus maximus, sartorius, adductor longus). The gracilis muscle transposition is the most commonly used technique. To prevent muscle fatigue and maintain constant contraction, this procedure involves the implantation of an electric pulse stimulator. The dynamic graciloplasty (stimulated gracilis muscle transposition) has yielded significant improvement in FI in 55% to 78% of patients [48-51]. This procedure is complex, associated with substantial morbidity, mostly infective complications, and no longer available in the United States.

The artificial bowel sphincter is an inflatable silicone cuff connected to a pump and a pressure-regulating balloon. The cuff is placed around the anal canal to maintain the basal pressure. The pump is then implanted in the scrotum or labia major. Deflation of the cuff with a shifting of fluid to the balloon, which is located behind the pubis, is controlled by the patient. One multicenter trial showed that the device was functional in 67% of patients during the first year after surgery. Despite a high rate of complications such as cuff erosion, infection in 25%, device failure and removal in 37%, and re-operations in 46%, the overall outcome is successful in more than 50% of patients[52].

Another innovative treatment of FI is sacral nerve stimulation (SNS). It stimulates both somatic and autonomic supply of the pelvic organs and anorectal region through the sacral and pudendal nerves with the neuromodulation of the sensory, motor, and autonomic innervation. However, the exact mechanism of action is unclear. Temporary electrodes are percutaneously implemented for 2-3 wk to stimulate the sacral nerve roots. If patients demonstrate significant improvement in incontinence, the leads are connected to a permanent stimulator in the subcutaneous tissue. Published results of this procedure are encouraging with marked improvement in continence in up to 100% and restored complete continence in 41%-75%[53-55]. Matzel et al published the results of a multicenter, prospective, nonrandomized trial in 37 patients with FI and showed functional improvement (reduction in incontinence episodes per week) in more than 80% of patients[54]. The SNS procedure may also be reliable in some patients with constipation.

Colostomy or ileostomy should be considered in patients with severe FI who have failed multiple procedures, are immobilized, or are considered high-risk. This procedure may be temporary to protect complex reconstructions and improve quality of life until complete distal healing, or permanent; many stomas are amenable to laparoscopic construction[56-58].

In conclusion, the management of OD is primarily medical and behavioral; biofeedback therapy is the preferred method of treatment. Except botulinum toxin injection for PPC, surgery is usually reserved for severe symptomatic patients, who have repairable anatomic defects or combined pathology. The treatment of FI should be based on the appropriate diagnosis, anorectal anatomic and physiologic investigation, and guided by the severity of incontinence. It is important to balance the anticipated improvement in the quality of life with a minimal acceptably low surgical morbidity. The first line of therapy, conservative treatment with biofeedback, is effective in the majority of patients. Well-defined external anal sphincter defects may be best repaired with sphincteroplasty, whereas radiofrequency, bulking agents, and SNS should be offered for suitable patients with more severe FI who have failed noninvasive therapy. Complex procedures such as gracilis muscle transposition and artificial bowel sphincter implantation are reserved for continued profound incontinence after failed previous surgery.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Gastrointestinal manifestations of endocrine disease

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Abstract

The hormonal interactions among the systems throughout the body are not fully understood; many vague clinical symptoms may in fact be manifestations of underlying endocrine diseases. The aim of the following review is to discuss gastrointestinal manifestations of surgically correctable endocrine diseases, focusing on abnormalities of thyroid function, cancer and finally autoimmune diseases. We also review manifestations of pancreatic endocrine tumors, and multiple endocrine neoplasia.

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Key words: Functional thyroid disease; Autoimmune thyroid disease; Thyroid malignancy; Islet cell tumors

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INTRODUCTION

Endocrine disorders are common, and the effects of endocrine disorders present with a wide range of clinical manifestations. This review encompasses the gastrointestinal manifestations of common endocrine diseases and the diagnostic and surgical interventions which may aid in the treatment of neuroendocrine abnormalities.

THYROID DISEASES

Functional thyroid disease

The true incidence of gastrointestinal manifestations of patients with functional abnormalities of the thyroid is not well documented. Gastrointestinal motor dysfunction, manifested by altered intestinal motility and transit time, has widely been accepted as the leading cause of gastrointestinal symptoms of thyroid disease[1]. The reported frequencies of gastrointestinal symptoms in hyperthyroid patients vary between 30% and 50%[2,3]. Patients with hyperthyroidism can experience frequent bowel movements, diarrhea, even malabsorption with steatorrhea[1,3]. Chronic dyspeptic symptoms such as epigastric pain and fullness, as well as eructation, nausea and vomiting are also frequently seen in these patients. Less commonly, hyperthyroidism has been reported to cause persistent and intractable vomiting[4]. The mechanism behind this is not clear; however, direct hormonal effects or stimulatory actions in the central nervous system have been suggested. Treatment of the thyrotoxicosis with beta-blocking agents and anti-thyroid drugs greatly improves these symptoms[4].

Dysphagia is a rare finding in thyrotoxicosis. The dysphagia can have either an acute or insidious onset, and may result from direct mechanical compression from a goiter, or altered neurohumoral regulation[5,6]. Clinically apparent symptoms vary with involvement of either the oropharyngeal or the esophageal phase of swallowing. There tends to be a higher incidence of oropharyngeal dysphagia than esophageal dysmotility. Clinically, these patients demonstrate easy drooling, nasal regurgitation, and weak phonation. They may have a higher incidence of aspiration pneumonia than do those patients with esophageal dysmotility. Dysphagia of thyrotoxicosis usually resolves shortly after resolution of the hyperthyroidism[5,6].

The overall decreased metabolic function seen in patients with hypothyroidism manifests in the GI tract with sluggish intestinal motility, ranging from mild constipation to paralytic ileus and colonic pseudo-obstruction[7]. Insidious symptoms of vague abdominal pain and distension may be present, and can be misdiagnosed as functional bowel disease. The exact biochemical mechanism by which thyroid disorders affect the gastrointestinal system are not fully understood. Changes may be seen on a molecular level with alteration of hormone receptors, dysfunction of the autonomic nervous system and myoelectrical enteric activity, as well as changes on a tissue level in the form of myopathy[3].

Altered gastroenteric myoelectrical activity has been documented by electrogastrography (EGG)[8]. Most studies in the literature detect a lack of correlation between the EGG and gastric emptying in non-euthyroid patients,

with manifestations of smooth muscle dysfunction, dissociation between the electrical signal and muscle response, pylorospasm, and lack of coordination between the antrum and the duodenum[9]. Hormonal effects on the GI tract may be a direct result of thyroid hormone, or as a result of synergistic effects of catecholamines. Tenore et al. investigated the effect of thyroxin (T4) on the intestinal chloride/bicarbonate exchange in hypo- and hyperthyroid rats. They found that alterations in intestinal ion exchange, mainly the flux of chloride, led to mucosal effects and resultant diarrhea. These findings were not seen when T4 was added ex vivo to rat ileum, suggesting that the effect on electrolyte transport likely requires systemic factors[10]. Furthermore, the beta-adrenergic antagonist propranolol inhibits intestinal transit in hyperthyroidism, thereby indicating that some of the dysmotility may be mediated through the adrenergic/catecholamine system[11]. Dysfunction of the autonomic nervous system (ANS) may modify the neuro-hormonal milieu, and result in alterations of myoelectric activity. However, there are no clear data regarding the exact mechanisms involved.

Hypothyroidism characteristically results in accumulation of glycosaminoglycans, mostly hyaluronic acid, in interstitial tissues throughout the body. This leads to interstitial edema that is particularly evident in the skin, heart muscle, and skeletal muscle, but also in the gastric smooth muscle. Edema in the gastric muscle may predispose to abnormalities of gastric myoelectric activity and thus the dysmotility seen in hypothyroid patients[12].

Thyroid malignancy

Patients with familial adenomatous polyposis (FAP), while known to be at high risk for colorectal cancer, also have an increased risk of thyroid malignancy. Papillary thyroid cancer (PTC) was first documented in a patient with FAP in 1949, and in 1968 a relation between FAP and PTC was suggested by Camiel *et al*[13,14]. The relative risk for PTC in FAP patients under 35 years of age has been found to be 160 times that of the general population. Thyroid cancer may be the first finding in patients with FAP, and 30% of thyroid carcinomas are diagnosed 4 to 12 years before the diagnosis of polyposis[15].

It has been suggested that FAP-associated thyroid tumors share unusual histological findings, such as the cribriform morular-variant of PTC (CMPTC)[16-20]. While the overall incidence of CMPTC in FAP patients has yet to be determined, analysis of large FAP registries suggests that it affects about 1%-2% of patients[20,21]. Similar to sporadic PTC, this disease demonstrates female predominance, and multifocality[22-26]. A recent series of seven such patients published by Tomoda et al demonstrated that all patients were female, with a mean age at presentation of 25 years[27]. Despite seemingly aggressive pathology, patients with CMPTC follow a highly favorable long-term course, with 5- and 20-year survivals of FAP-associated CMPTC reported to be 90% and 77%[16]. Patients diagnosed with CMPTC should be screened for FAP[27].

Autoimmune thyroid disease (AITD)

AITD, such as chronic lymphocytic thyroiditis (Hashimoto's thyroiditis) and Graves' disease, has been linked to autoimmune disorders of the gastrointestinal tract, namely celiac disease and atrophic gastritis. This association is not surprising considering that these disease entities share a common haplotype, HLA DR3-DQ2, which is common to many autoimmune diseases[28,29].

Celiac disease, a gluten-induced small bowel mucosal inflammation, demonstrating crypt hyperplasia and villous atrophy, is associated with an increased frequency of AITD. Patients with AITD are affected by celiac disease 5-10 times more than the general population. Also, 4%-10% of patients with celiac disease will also manifest AITD. Positive anti-thyroid antibodies can be seen in 10%-15% of patients with celiac disease. The significance of this discovery of subclinical thyroiditis is uncertain. Patients with AITD who remain hypothyroid despite adequate thyroid hormone supplementation, should undergo screening for celiac disease, as mucosal abnormalities may preclude full absorption of levothyroxine[30].

Atrophic gastritis is a chronic disease characterized by disappearance of oxyntic glands, loss of production of hydrochloric acid and intrinsic factor, as well as demonstration of parietal cell autoantibodies (PCA)[31,32]. Autoimmune gastritis is an early event in juvenile patients with AITD and detectable PCA. The lack of gastric symptoms in this young population often leads to a delay in diagnosis, thus screening for PCA with biochemical markers of gastric damage, such as gastrin levels, is warranted. Abnormal levels help to select patients who may benefit from further investigation with gastroscopy. Segni et al, determined that 30% of juvenile patients with AITD had positive PCA, and demonstrated that 45% of these patients had mild to severe hypergastrinemia[33]. Screening of juvenile patients with AITD is warranted, as atrophic gastritis is associated with a higher risk of gastric cancer and carcinoid tumors[34,35]. Premalignant gastric lesions can be found in up to 26% of these relatively young patients with detectable PCA[36].

ISLET CELL TUMORS OF THE PANCREAS

Islet cell tumors of the pancreas are rare entities which can range in presentation with respect to functionality, malignancy, and pattern of inheritance. The first descriptions of endocrine tissue in the gut came during the late 1800's with the recognition of 'islands' of cells in the pancreas by Langerhans, followed later by the discovery of chromaffin cells in the stomach by Rudolf Heidenhain, and then in the intestinal mucosal crypts of Lieberkuhn by Nikolai Kulchitsky. The recognition and intermittent reporting of islet cell adenomas began in 1902. However it was not until 1921 that the first reported clinical implication of these adenomas became known. Over the next 50 years several different peptides secreted by these tumors were identified, and eventually they were recognized as amine precursor uptake and decarboxylation (APUD) cell lesions, and coined apudomas. Based on functionality, these lesions were classified into clinical syndromes[37].

The clinical presentation of patients with pancreatic endocrine tumors varies from vague complaints of

abdominal pain and nausea to debilitating diarrhea. Due to the variability in clinical symptoms, a biochemical diagnosis is necessary. Once a definitive diagnosis is made based on serum markers, localization often necessitates multiple imaging modalities. Ultrasound is often the first test utilized, but has a low sensitivity, up to 31%[38-40]. Computed tomography (CT) and magnetic resonance imaging (MRI) technologies have improved sensitivity for small lesions, up to 40%-70%[38-41]. In cases where conventional imaging fails to adequately localize the lesion, invasive testing, such as intra-arterial stimulation and venous sampling, provides additional data. While these procedures can be invasive and technically challenging, the results are reliable and specific[42,43].

Labeled octreotide scanning is useful, as many islet cell tumors demonstrate type 2 somatostatin receptors[40]. Positron emission tomography utilizing a serotonin precursor 5-hydroxy-tryptophan has shown excellent results in a preliminary study from Eriksson and colleagues[44]. Endoscopic ultrasound has rapidly come to the forefront in localization studies. While operator dependent, it is highly sensitive in detecting even small tumors. Limitations include tumors in the distal pancreas and those that are pedunculated. In addition, fine needle aspiration can be preformed at the same time for both diagnosis and tattooing of the lesion to allow easy identification at the time of resection[40].

The operative approach varies on the type and extent of tumor. Enucleation is the procedure of choice for benign tumors, while malignant lesions require en bloc resection due to local invasion or presence of metastases. Laparoscopy is appropriate in some cases where enucleation or distal pancreatectomy is required[45].

Insulinoma

Insulinoma is the most common islet cell tumor. It was first described by Wilder and W. J. Mayo in 1927[37]. This tumor is typically solitary, small, and benign. It may be sporadic or associated with MEN I[40]. Hyper-secretion of insulin is often associated with the clinical findings of the "Whipple's triad", which consists of symptoms and signs of hypoglycemia during times of fasting or exertion, a serum glucose level less than 2.5 mmol/L during these symptoms, and resolution of the symptoms with administration of glucose[40,46]. Documenting elevated insulin levels during hypoglycemic periods, levels of endogenous insulin secretion with a C-peptide measurement and calculating the insulin:glucose ratio confirm the diagnosis. Adenomas, islet cell hyperplasia or nesidioblastosis can be the cause of hyperinsulinemia. The adenomatous lesions tend to be small and may be difficult to localize pre-operatively. Localization should be performed with conventional imaging including endoscopic US (EUS), CT, MRI, octreoscan, as well as calcium stimulated arterial-venous sampling (Doppman-Imamura test) for regionalization, if needed. Intraoperative ultrasound is extremely sensitive in localizing small lesions with a sensitivity of 91% in one series[47].

Enucleation is the preferred surgical treatment. In patients with diffuse disease, or tumors too small to localize, a regional resection is warranted after the general location of the tumor is identified by pre-operative arterial stimulation and venous localization[38,43]. Blind resection is not recommended.

Gastrinoma

Zollinger and Ellison described the gastrinoma syndrome (ZES) in 1955[37]. ZES includes a tumor associated with hypergastrinemia, peptic and distal duodenal ulcers. This is the most common islet cell tumor in MEN I, seen in up to 40% of patients. Although considered an islet cell tumor, this lesion is more frequently located in the duodenum, and can arise anywhere within the gastrinoma triangle, which is bounded by the confluence of the cystic and hepatic bile ducts, the junction of the 2nd and 3rd portions of the duodenum, and the neck of the pancreas. The rate of malignancy was noted to be 52% in one of the largest published series by Norton *et al*[48]. Clinically, patients present with recurrent ulcer disease, abdominal pain, diarrhea, and melena; they all demonstrate hypergastrinemia. Symptoms can be controlled with proton pump inhibitors to provide and opportunity to localize the lesion. Imaging with CT and MRI can be diagnostic; EUS has also proven useful as the majority of these tumors are located in the wall of the duodenum. Difficult localization or regionalization can be obtained by visceral arterial stimulatory testing using either secretin or calcium as a secretogogue, with hepatic venous sampling[38,39,42,48,49].

In those lesions that are difficult to localize pre-operatively, a thorough search must be undertaken in the gastrinoma triangle intra-operatively. Intraoperative endoscopy for identification of small intramural duodenal adenomas can be helpful. A duodenotomy is performed to allow clear evaluation of the wall of the duodenum and resection of lesions. If no obvious abnormality can be found, then a pancreaticoduodenectomy may be performed[40]. The role of resection of liver metastases for gastrinoma is not well defined at the present time. Debulking of > 90% of tumor burden may help in overall palliation of symptoms, and may be accomplished surgically or in combination with radiofrequency ablation and/or cryotherapy ablation[40].

VIPoma

These rare tumors, described by Verner and Morrison in 1958, are defined by the oversecretion of vasoactive intestinal peptide (VIP)[37]. The clinical syndrome is one of voluminous secretory watery diarrhea, with hypokalemia and hypochlorhydria. The profound diarrhea leads to significant losses of chloride and potassium, producing symptoms of incapacitating muscle weakness[46,51]. Other symptoms include flushing, due to vasodilatory effects of VIP, hyperglycemia due to glycogenoloytic activities of VIP, and hypercalcemia. The triad of severe secretory diarrhea, elevated fasting levels of VIP, and identification of an islet cell tumor is diagnostic. In a study performed by Koch *et al*[52] the determination of VIP levels can differentiate between patients with chronic diarrhea of other sources, patients with a non-functional islet cell tumor and those due to a functional tumor. VIP levels in patients with a functional tumor were 160-5975 ng/L compared to 80-120 ng/L in those with a non-functional

tumor. Most patients produce diarrhea in excess of 5 L/d, but no less than 0.7 L/d. Localization can be accomplished with conventional imaging, as both CT scan and MRI are sensitive tests for pancreatic and liver lesions. Octreoscan is useful in individuals with equivocal imaging. The most common location for this tumor is the tail of the pancreas, and a majority of these patients will have liver metastases at diagnosis[51].

Octreotide reduces circulating levels of VIP and allows for improvement of the diarrhea. After preoperative optimization, surgical exploration for resection is the most effective treatment. As a significant portion of these patients will have malignant disease at the time of exploration, careful examination of the liver and lymph nodes is necessary. Debulking may have a benefit in improving the symptoms by lowering VIP levels[40,46]. In cases of unresectable disease, adjuvant therapy with octreotide, streptozotocin, 5-fluorouracil and doxorubicin can palliate the symptoms[40]. However, a portion of patients may not respond to octreotide, or may become refractory to the drug due to down-regulation of receptors. Mean survival is 3.6 years, with a possible long-term survival of up to 15 years[51].

Glucagonoma

Glucagonoma was identified by McGarvran *et al*[37] in 1966. While rare, this islet cell tumor may have the most distinctive clinical syndrome. It arises from cells resembling alpha islet cells, is the third most common functioning endocrine tumor, and may be both sporadic or associated with MEN I[40]. Symptoms of excessive glucagon secretion are manifested by a migrating skin rash, diabetes, malnutrition, weight loss, anemia, thrombophlebitis, and glossitis. The necrolytic migratory erythema that is pathognomonic for this condition is felt to be secondary to hypoaminoacidemia associated with elevated levels of glucagons. Restoration of serum amino acids may alleviate this condition. However the data are contradictory. Malignancy is common with these tumors, and the prognosis is worse than with other types of islet cell tumors. The most common age at diagnosis is between 50-60 years. Patients often present with the characteristic pruritic skin rash located on the lower extremities and groins, glucose intolerance or frank diabetes mellitus. Thromboembolic disease is common, and can be the cause of death in some patients[46].

Biochemical diagnosis is based on elevated levels of plasma glucagon. Localization can be accomplished with CT scanning, as these tumors are typically large and located in the distal pancreas. Hepatic metastasis at the time of diagnosis is common[40]. Preoperative preparation involves control of hyperglycemia, treatment of thrombosis, and optimization of nutritional status with total parenteral nutrition. Octreotide has occasionally been used to aide in reduction of plasma glucagons levels, allowing improvement of nutritional status, and alleviation of the rash[46].

An aggressive operative approach is the mainstay of treatment. En bloc resection is required due to the high risk of malignancy, and liver metastases should be resected at the same setting if possible. Hepatic artery embolization, chemotherapy with 5-fluorouracil and streptozotocin, as well as long-term octreotide may be adjunctive. Liver and pancreatic transplantation have been performed in rare instances. The metastatic disease can be indolent, leading to survival for many years[46].

Somatostatinoma

Identification of somatostatinoma was made by Larssen *et al* and Ganda *et al* in 1977[37]. These rare tumors are frequently located in both the duodenum and pancreas, and secrete excess somatostatin. Overproduction of this inhibitory peptide leads to vague symptoms, marked by a decrease in gastrointestinal function. Clinically, it may present with cholelithiasis (70%), mild diabetes (60%), steatorrhea (30%-68%), hypochlorhydria (33%-53%) and weight loss[46]. Many of these tumors are identified incidentally on imaging studies for vague abdominal complaints, or at the time of cholecystectomy. The biochemical diagnosis is made by increased serum levels of somatostatin. Most of these tumors are located in the head of the pancreas or duodenum, and are usually solitary. Pancreaticoduodenectomy is often necessary due to the high risk of malignancy[40].

OTHER FUNCTIONAL AND NON-FUNCTIONAL NEUROENDOCRINE TUMORS

Islet cell tumors of the pancreas can secrete a variety of other hormones, including pancreatic polypeptide, grhelin, corticotropin, neurotensin, and calcitonin[46,51]. The majority of these tumors produce vague symptoms of abdominal discomfort and diarrhea. They can be difficult to diagnose, and should be approached in a similar manner to other functional islet cell tumors[40,46].

Tumors that produce peptides not associated with a clinical syndrome are considered to be non-functional. These lesions often produce symptoms associated with mass effect, such as abdominal discomfort or pain, nausea, emesis, obstruction, cachexia, bleeding, and obstructive jaundice[54]. Due to the lack of functional symptoms, these tumors are typically large when diagnosed, and have been noted to average 10 cm in two published series[54,55]. Conventional imaging with CT will identify these tumors, but they are difficult to distinguish from pancreatic adenocarcinoma. Operative approach to these lesions should be with intent of curative resection by pancreaticoduodenectomy, or distal pancreatectomy. If the lesion cannot be safely resected, an open biopsy should be obtained prior to palliation with gastrointestinal bypass. In a large series published by Liang *et al*, adjuvant chemotherapy and radiation therapy provided a disease-free survival of up to 45 mo, with an overall 5 and 10 year survival of 58% and 29%[54].

Multiple endocrine neoplasia syndromes (MEN)

MEN 1 is characterized by pituitary lesions, islet cell tumors of the pancreas, and hyperparathyroidism. The MEN 1 pancreas can manifest any of the previously mentioned tumors, the most common being gastrinoma. The pancreatic pathology can be in the form of a macroadenoma, microadenoma, islet cell hypertrophy,

hyperplasia, dysplasia, and carcinoma; approximately 50% of MEN 1 patients have tumors which are duodenal in origin. It has been shown that early detection can improve long-term survival, therefore early screening with pancreatic polypeptide and gastrin is advocated for family members of MEN 1 patients[46]. In addition, measurement of insulin, proinsulin, glucagon, and chromogranin A can be utilized. If two of these markers increase over the course of 6 mo, further investigation should be undertaken, and those patients should be referred for surgical exploration[56]. Cure for gastrinoma is difficult due to the size and multiplicity of lesions. The largest series of surgical resection and cure comes from the University of Michigan in which and an 80% pancreatectomy, thorough exploration of the duodenum, and enucleation of smaller pancreatic lesions has been performed[46]. Total pancreatectomy, pancreaticoduodenectomy and pancreas-preserving total duodenectomy have also been reported[46,57].

MEN 2 is subdivided into MEN 2A, characterized by medullary thyroid carcinoma (MTC), pheochromocytoma, and parathyroid hyperplasia; and MEN 2B which includes MTC, tumors of the adrenal medulla (pheochromocytoma, and/or ganglioneuroma), intestinal and mucosal neuromas, and marfanoid habitus. These syndromes can manifest a host of GI symptoms, including diarrhea associated with hypercalcitonemia of MTC, constipation associated with hypercalcemia, and most notably, Hirschsprung's disease associated with MEN 2B[46]. Ganglioneuromas of the intestinal mucosa can lead to manifestations of Hirschsprung's disease with significant symptoms of constipation, pain, distension, and difficulty swallowing. The majority of these patients require surgical intervention for their colonic disease[58].

In summary, endocrine disorders of the thyroid and pancreas often have significant gastrointestinal symptoms. Given the often vague and general quality of these symptoms, diagnosing their endocrine etiology requires a high index of suspicion. Treatment can be achieved both medically, and surgically, if indicated or feasable.

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TOPIC HIGHLIGHT

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Less common neoplasms of the pancreas

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Abstract

Recently, there has been an increased recognition of neoplasms of the pancreas other than ductal adenocarcinoma. Although not as well studied or characterized as pancreatic adenocarcinoma there are many distinct lesions which exhibit diverse biological behaviors and varying degrees of malignancy. These lesions include: endocrine neoplasms, cystic tumors, solid pseudopapillary tumors, acinar cell carcinoma, squamous cell carcinoma, primary lymphoma of the pancreas, and metastatic lesions to the pancreas. These less common neoplasms are being diagnosed more frequently as the number and sensitivity of diagnostic imaging studies increase. This review article discusses the clinical course, diagnosis, and treatment of these less common, but quite relevant, neoplasms of the pancreas.

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INTRODUCTION

Ductal adenocarcinoma is the most common of the various tumors that affect the pancreas. There are other distinctive neoplasms of the pancreas, however, which display a wide range of symptoms, biological behaviors, and outcomes. These include endocrine neoplasms, cystic tumors, solid pseudopapillary tumors, acinar cell carcinoma, squamous cell carcinoma, pancreatic lymphoma, and metastatic lesions of the pancreas. These tumors are becoming more frequently documented in the literature and recognized more often, and at earlier stages, due to the increased sensitivity and utilization of diagnostic imaging. Therefore, the correct identification and treatment of these less common neoplasms of the pancreas is becoming increasingly important.

ENDOCRINE NEOPLASMS OF THE PANCREAS

Pancreatic endocrine tumors are far less prevalent than pancreatic adenocarcinomas. They occur in approximately 1 out of 100 000 people annually, and constitute 1%-2% of pancreatic tumors[1]. These tumors frequently manifest as a constellation of symptoms associated with the hormones they produce (Table 1). Classification is based on hormone production and the functional pancreatic endocrine tumors include: insulinomas, gastrinomas, VIPomas, glucagonomas, somatostatinomas, adrenocorticotropic hormone producing tumors, and growth hormone releasing factor secreting tumors. Additionally, there is a subset of non-functional pancreatic tumors which are clinically inert but produce neuroendocrine cell markers such as chromogranin-A and somatostatin receptors[2]. Pancreatic endocrine tumors are also associated with several inherited syndromes, including multiple endocrine neoplasia type I (MEN-1) and von Hipple Lindau disease (vHL).

Insulinomas are the most common functional pancreatic neuroendocrine tumor and are most often associated with fasting hypoglycemia and neuroglycopenic symptoms but may present with cardiovascular symptoms as well. These tumors are frequently small (< 2 cm), predominantly benign, and may occur anywhere throughout the pancreas[3]. The second most common functional pancreatic endocrine tumors are gastrinomas. These tumors produce gastrin and cause Zollinger-Ellison syndrome. Greater than 50% of gastrinomas are malignant at the time of diagnosis and many of these will have liver metastases[4]. These patients commonly have peptic ulcer disease but diarrhea secondary to the increased gastric acid secretion is frequently a presenting symptom[5]. Other less common functional neuroendocrine tumors of the pancreas include VIPomas which are frequently metastatic at the time of diagnosis[6] (> 60%) and cause secretory diarrhea with subsequent electrolyte abnormalities. Glucagonomas are another rare functional pancreatic endocrine tumor. They are usually large (> 4 cm) tumors found almost exclusively within the body and tail of the pancreas and are symptomatically associated with the "4D's" (dermatitis, diabetes, deep venous thrombosis, and depression)[7]. There are additional

Table 1 Classification of pancreatic endorcrine tumous

Tumor	Hormone produced	Percent malignant	Clinical features	5 yr survival	Localization	Treatment
Insulinoma	Insulin proinsulin	10%	Hypoglycemia, neuroglycopenic symptoms	97%	EUS, intraoperative ultrasound	Surgical resection, chemotherapy, dextrose
Gastrinoma	Gastrin	60%-90%	Peptic ulcer disease, diarrhea	60%-70%	SRS, SPECT, EUS	Surgical resection, PPI, somatostatin
Glucagonoma	Glucagon	50%-90%	Necrolytic migratory erythema, diabetes, DVT, depression	50%-60%	CT w/contrast, EUS	Surgical resection, nutritional support, somatostatin, chemotherapy, insulin
VIPoma	Vasoactive intestinal peptide	40%-70%	Secretory diarrhea, hypokalemia, achlorhydria	50%	CT Scan, SRS	Somatostatin, correction of electrolytes, hydration, surgical resection
Somatostatinoma	Somatostatin	70%-80%	DM, cholelithiasis, steatorrhea	40%	CT, EUS, SRS	Surgical resection chemotherapy, somatostatin
Non-functional tumors	Pancreatic polypeptide	60%-80%	Abdominal pain, weight loss, jaundice	50%	CT, MRI	Surgical resection

functional neuroendocrine tumors of the pancreas, each of which present with specific clinical symptoms related to the excess of hormone produced. However, approximately 15%-30% of pancreatic endocrine tumors are termed "nonfunctional", as they may either produce elevated amounts of hormones which do not cause symptoms or may excrete small, clinically insignificant amounts of active hormones[3]. These tumors are generally large, metastatic at the time of diagnosis, and may be distributed throughout the pancreas. Patients generally present with mechanical symptoms associated with the tumor itself, such as abdominal pain, weight loss, and jaundice[8].

The treatment of endocrine tumors of the pancreas includes supportive medical treatment, surgery, both for cure and palliation, and nonsurgical cytoreduction. Frequently, these tumors require a combination of both surgical and medical treatment to best relieve the patients' symptoms and offer the best prognosis. It is important to remember that endocrine neoplasms of the pancreas usually have metabolic derangements which must be corrected prior to further treatment. Patients with insulinomas should have their hypoglycemia controlled, aggressive preoperative nutritional support and anticoagulation is essential for those with glucagonomas, and dehydration and electrolyte abnormalities must be corrected when a VIPoma is diagnosed.

Complete surgical resection of endocrine neoplasms of the pancreas offers the only chance for cure, but patients can sometimes have metastases at the time of diagnosis making complete resection an impossibility. However, some authors have suggested that in patients with metastatic disease, aggressive surgery with debulking and resection of metastases will benefit patients symptomatically and improve survival[9,10]. Combinations of non-surgical cytoreductive modalities and medical treatments are also used to enhance palliation and survival, including cryoablation, hepatic artery embolization, radio frequency ablation, somatostatin analogues, alpha interferon, chemotherapy, and radiotherapy[11].

CYSTIC NEOPLASMS OF THE PANCREAS

The diagnosis of cystic lesions of the pancreas, which constitute less than 10 percent of pancreatic neoplasms[12], has become more commonplace due to the increased frequency and improved accuracy of imaging techniques. This has increased the need for a clear understanding of the diagnosis and management of these cystic lesions. Two of the more common cystic neoplasms are the serous cystadenomas and the mucinous cystic neoplasms. Serous cystadenomas occur predominantly in women, and are typically described as microcystic with a "honeycombed" appearance, although oligo-macrocystic variants do exist. All true serous cystadenomas are benign. However there are a few case reports in the literature of serous cystadenocarcinomas which are classified as malignant due to invasion of cells into surrounding tissues or metastatic spread[13].

Mucinous cystic neoplasms (MCNs) have an epithelial lining made of columnar mucin-producing epithelium that has an ovarian-type stroma and stains positively for estrogen and progesterone receptoms[14]. Unlike the honeycombed appearance of serous neoplasms, most mucinous tumors are unilocular and although uncommon, a finding of peripheral calcification on CT is predictive of a malignant MCN[15]. Series have found that from 8% to 33% of these tumors contain invasive carcinoma[16]. MCNs are generally found in the body and tail of the pancreas and more commonly affect females in the 5th decade of life. With both neoplasms, patients often present with nonspecific complaints such as abdominal pain and weight loss, though they may be asymptomatic[17]. Of note, in a recent review of 106 patients with serous adenomas, 47% of tumors were found in asymptomatic patients as part of the workup of an unrelated problem[18]. It is this type of patient with an incidentally found lesion that presents a challenge in both diagnosis and treatment. Given the risk of invasive cancer the treatment of mucinous cystic neoplasms should be surgical resection. The treatment of serous cystic lesions is more controversial. A recent paper from the Massachusetts General Hospital describing their experience with serous cystadenomas proposes a selective approach towards resection, based on tumor size and the ability to accurately diagnosis the lesion[18]. If radiographic imaging is equivocal, endoscopic ultrasound with fine needle aspiration is performed and markers such as mucin-like antigen and carcinoembryonic antigen (CEA) levels, which are both low in serous tumors and high in

mucinous lesions, are evaluated[16]. If based on this workup the diagnosis of a serous cystadenoma of < 4 cm in an asymptomatic patient is made, expectant management with serial CT scans is recommended[18]. However, due to the difficulty in unequivocally differentiating between benign versus malignant cystic tumors preoperatively, some feel that an aggressive surgical approach should be used for all patients presenting with a cystic pancreatic neoplasm[19,20].

INTRADUCTAL PAPILLARY MUCINOUS TUMORS

Intraductal papillary mucinous neoplasms (IPMNs) of the pancreas are defined as intraductal mucin-producing neoplasms with tall, columnar, mucin-containing epithelium with or without papillary projections. Typically, these tumors involve the main pancreatic duct but recently, a distinction has been made between those tumors which involve the main duct and those which predominantly involve the branches of the ductal system (branch-duct IPMNs). Some have suggested that the branch duct variant of IPMN is more indolent and has a lesser incidence of malignancy than the main duct IPMN[21,22]. IMPNs display a histological range from adenoma, to IMPN with dysplasia, to carcinoma in situ, to invasive carcinoma which seems to mimic the adenoma-carcinoma sequence seen in colorectal cancer. A review of recent reports found that approximately 50% of resected specimens contained invasive carcinoma[23]. Patients generally present in their 60's, but several recent studies have shown that those patients with invasive IPMNs are 5-6 years older than those patients with benign disease[24,25]. Patients are often symptomatic at presentation with complaints of abdominal pain, weight loss, and diarrhea[26]. Thin cut CT scan, which is the radiological test of choice, will often reveal a mass in the head of the pancreas and if diagnostic uncertainty is present, endoscopic ultrasound with fine needle aspiration may be preformed. Treatment of IPMN consists of surgical resection, often a pancreaticoduodenectomy, of all gross disease with an attempt to achieve negative surgical margins. A comparison of recent reports showed a 5 year survival rate from 77%-100% in noninvasive IPMNs compared to 43%-65% in those patients with invasive disease[23-27]. Recurrent disease has been shown to occur both in the pancreatic remnant and at distant sites and therefore close follow-up with frequent imaging has been recommended.

SOLID PSEUDOPAPILLARY TUMORS OF THE PANCREAS

These rare pancreatic neoplasms tend to affect young women and have characteristic histological features, including a combination of solid and cystic components and the presence of pseudopapillae. Interestingly, unlike ductal adenocarcinoma, solid pseudopapillary tumors of the pancreas (SPTs) are not associated with abnormalities in K-*ras*, p53, or DPC4 genes but like colon cancer, do demonstrate genetic abnormalities in the APC/*B*-catenin pathway[28]. Presenting symptoms are frequently non-specific in nature and often include abdominal pain, increased abdominal girth, and the presence of a palpable mass. A review of 718 patients with solid pseudopapillary tumors of the pancreas found a 10:1 ratio of affected women to men, a mean age of 22 years, the presence of metastases in 20% of the patients, and a cure rate greater than 95%[29]. Surgery is the treatment of choice for these tumors, with pancreaticoduodenectomy performed for lesions in the head of the pancreas and distal pancreatectomy for those lesions in the body or tail. Metastatic or locally invasive disease is not necessarily a contraindication to surgery, and improved longterm survival has been demonstrated with aggressive surgical resection[30,31]. There have been reports of some success with radiotherapy or chemotherapy in cases deemed unresectable[29]. Although the majority of these tumors are considered to be of low malignant potential, a recent report of two cases of aggressive SPTs noted death of the patients at 6 and 16 mo after diagnosis. In these cases a high mitotic index and extensive tumor necrosis was noted[32]. These findings might help distinguish a more virulent form of SPT and lead to a more aggressive post-operative treatment course.

ACINAR CELL CARCINOMA

Acinar cell carcinoma of the pancreas accounts for approximately 1% of pancreatic cancers. These tumors exhibit expression of pancreatic enzymes such as trypsin, lipase, chymotrypsin and amylase. Similar tumors with primarily acinar differentiation include mixed acinar-endocrine carcinomas, which exhibit both acinar and endocrine cellular differentiation, and pancreatoblastomas which are usually found in children. Patients most frequently present with abdominal pain and bloating and these tumors may be associated with hypersecretion of lipase which leads to subcutaneous nodules and ectopic fat necrosis[33]. On CT or MRI it presents as an exophytic, hypovascular, and well marginated mass[34]. Unlike typical pancreatic adenocarcinoma the genetic mutations in K-*ras*, *p*53, and S*mad* are uncommonly found but there seem to be alterations in the adenomatous polyposis coli-*b*-catenin pathway which are similar to colorectal cancer[35].

A retrospective review from the Memorial Sloan-Kettering Cancer Center evaluated 39 patients with acinar cell carcinoma[36]. They noted that these lesions were found equally throughout the pancreas, approximately half the patients had metastatic disease at time of presentation, and that the median survival was 19 mo. This median survival (19 mo) falls between that of ductal adenocarcinoma (6 mo) and endocrine neoplasms of the pancreas (40-60 mo). In these patients the stage of disease correlated with survival (14 mo in patients with metastases *vs* 38 mo in patients with localized disease). Patients who were amenable to surgery had a 36 mo median survival as opposed to 14 mo in patients who did not receive surgery. Despite the improved survival in the surgical patients, there was a high recurrence rate (72%) which the authors felt may have been indicative of the presence of micrometastases and may imply the need for adjuvant therapy. Many of these patients who recurred experienced a distant rather than lo-

cal recurrence which suggests the need for better systemic therapy. Case reports have demonstrated some success with concurrent chemotherapy (capecitabine or irinotecan, FU and leucovorin) and radiotherapy which will likely be further elucidated as more data is obtained[36,37].

SQUAMOUS CELL CARCINOMA OF THE PANCREAS

Although not normally present in the pancreas, squamous metaplastic cells may present during periods of inflammation[38]. These cells may then transform to squamous cell carcinoma of the pancreas which is a rare and unusual cancer. The reported incidence is 0.5% to 2% of pancreatic malignancies and a recent case report found 61 cases, including 36 autopsy/registry cases, in the English literature[39]. Their review of these cases found a mean age of 62 years, common presenting symptoms of abdominal/back pain, weight loss, and anorexia, tumor size of 7.8 cm, and equal distribution of the lesion in the head, body, or tail[39]. Given the rarity of this neoplasm, in cases in which squamous cell carcinoma of the pancreas is diagnosed it is important to perform a thorough search for a possible alternate primary location. Patients with squamous cell carcinoma of the pancreas have a poor prognosis, with a median survival of 7 mo for patients undergoing curative resection and 3 mo for those not undergoing a curative resection[39,40]. There are several reports of the use of chemotherapy in unresectable cases, but the prognosis remains poor.

PRIMARY LYMPHOMA OF THE PANCREAS

Primary lymphoma of the pancreas is rare disease and in one study was shown to comprise 1.3% of 1050 pancreatic fine needle aspirations[41] Patients generally range from 58-66 years old and frequently present with abdominal pain, weight loss, and an abdominal mass, while only 25% present with jaundice[42]. The head of the pancreas is the most common location for these tumors but the entire gland may be affected. The majority of primary pancreatic lymphoma displays a B-cell phenotype, although a Japanese report found that 21% of cases were T-cell lymphomas and cases of anaplastic large cell lymphoma and Hodgkin lymphoma of the pancreas have been reported[43-46]. Diagnosis of this rare cancer is critical and requires adequate samples for tissue typing. Fine needle aspiration (FNAs) with flow cytometry has been advocated by some to be an accurate diagnostic tool which then obviates the need for invasive surgery to only those patients with non-diagnostic FNAs[47]. The primary treatment of pancreatic lymphoma consists of chemotherapy and radiotherapy using non-Hodgkin's lymphoma protocols and survival ranges from to 2-6.5 years[42]. For patients undergoing surgery, biliary and gastric bypass may be beneficial to relieve jaundice and prevent obstructive fibrosis in the common bile duct following chemotherapy and radiation. A review of those patients undergoing surgery found that 35% of patients underwent biopsy alone, 27.5% had a biliary bypass, 15% had a gastric bypass, and 7.5% had pancreatic resection[42].

METASTATIC LESIONS OF THE PANCREAS

Although uncommon, the pancreas may serve as a site for distant metastases and when these metastases present as an isolated pancreatic mass, questions regarding diagnosis and treatment may arise. Almost any tumor type may metastasize to the pancreas but in an analysis of a surgical and autopsy database, the majority of tumors found in the surgical database were lymphomas, carcinomas of the stomach, kidney, and lung whereas in the autopsy database metastasis was most commonly from the lung, followed by the GI tract and kidney[48]. A review of resection for metastases to the pancreas found renal cell carcinoma to be the most frequent primary histopathology (62%), followed by non-small cell lung cancer, melanoma, and sarcoma[49]. In this series, postoperative morbidity was 25%, mortality was 6%, and the overall actuarial survival rate for 2 and 5 years was 62% and 25%, which indicates that resection of metastatic disease to the pancreas is safe and may offer some survival benefit in select patients. The prolonged disease free interval between the initial cancer and pancreatic recurrence can be quite prolonged (median 2.6-7.5 years)[48-51]. Hiotis *et al* found that approximately one-third of the metastatic tumors evaluated were clinically suspected to be primary pancreatic neoplasms[49]. These findings demonstrate the difficulty in diagnosing pancreatic metastases and emphasize the need to consider metastatic disease to the pancreas in the workup of any pancreatic tumor and particularly in patients with a history of prior malignancy.

In conclusion, the most common neoplasm affecting the pancreas is ductal adenocarcinoma but there are a host of less common pancreatic neoplasms with distinct clinical and biological behaviors. Although relatively rare, more of these lesions will be seen as diagnostic imaging continues to become more frequent and sensitive. Accurate and timely identification of a pancreatic neoplasm coupled with a knowledge of its biological behavior and therapy will be essential in order to offer patients the optimal therapy.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Modern management of rectal cancer: A 2006 update

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Abstract

The goal of this review is to outline some of the important surgical issues surrounding the management of patients with early (T1/T2 and N0), as well as locally advanced (T3/T4 and/or N1) rectal cancer. Surgery for rectal cancer continues to develop towards the ultimate goals of improved local control and overall survival, maintaining quality of life, and preserving sphincter, genitourinary, and sexual function. Information concerning the depth of tumor penetration through the rectal wall, lymph node involvement, and presence of distant metastatic disease is of crucial importance when planning a curative rectal cancer resection. Preoperative staging is used to determine the indication for neoadjuvant therapy as well as the indication for local excision versus radical cancer resection. Local excision is likely to be curative in most patients with a primary tumor which is limited to the submucosa (T1N0M0), without high-risk features and in the absence of metastatic disease. In appropriate patients, minimally invasive procedures, such as local excision, TEM, and laparoscopic resection allow for improved patient comfort, shorter hospital stays, and earlier return to preoperative activity level. Once the tumor invades the muscularis propria (T2), radical rectal resection in acceptable operative candidates is recommended. In patients with transmural and/or node positive disease (T3/T4 and/or N1) with no distant metastases, preoperative chemoradiation followed by radical resection according to the principles of TME has become widely accepted. During the planning and conduct of a radical operation for a locally advanced rectal cancer, a number of surgical management issues are considered, including: (1) total mesorectal excision (TME); (2) autonomic nerve preservation (ANP); (3) circumferential resection margin (CRM); (4) distal resection margin; (5) sphincter preservation and options for restoration of bowel continuity; (6) laparoscopic approaches; and (7) postoperative quality of life.

INTRODUCTION

Rectal cancer is a major health concern in the United States, with an estimated 40 340 new cases diagnosed in 2005[1]. There are four major goals in the treatment of a patient with rectal cancer: (1) local control; (2) long-term survival; (3) preservation of anal sphincter, bladder, and sexual function; and (4) maintenance or improvement in quality of life. These goals are best achieved through a multi-modality approach delivered by a multi-disciplinary team. The aim of this review is to outline some of the important surgical issues surrounding the management of patients with early (T1/T2 and N0), as well as locally advanced (T3/T4 and/or N1) rectal cancer.

PREOPERATIVE STAGING

Information concerning the depth of tumor penetration through the rectal wall, lymph node involvement, and presence of distant metastatic disease is of crucial importance when planning a curative rectal cancer resection. Preoperative staging is used to determine the indication for neoadjuvant therapy, as well as to determine whether local excision or radical cancer resection will provide optimal surgical treatment.

The most common imaging modalities currently used in the preoperative staging of rectal cancer are endorectal ultrasound (ERUS), computed tomography (CT) scan, magnetic resonance imaging (MRI), and positron emission tomography (PET) scan.

ERUS

ERUS is an accurate method to preoperatively stage rectal cancers. Although operator-dependent, it can be performed at the time of patient evaluation with minimal preparation or patient discomfort. ERUS is used to clinically determine the tumor (T) and lymph

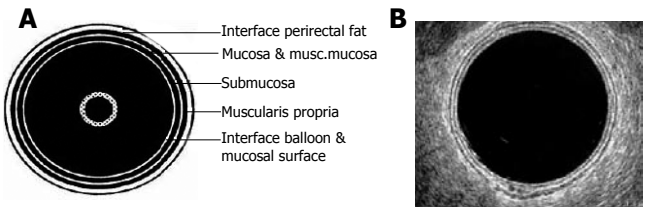


Figure 1 Rectal wall anatomy. **A**: Schematic diagram of ERUS image; **B**: Actual image of normal ERUS[3].

node (N) stage of rectal cancer (Figure 1). According to currently available data, ERUS is 62% to 92 % accurate for T-staging and 64% to 88% accurate for N-staging[2,3]. ERUS is limited by its steep learning curve, operator variability, and limitations for staging near-obstructing tumors and downstaged tumors after chemoradiation therapy. Nevertheless, ERUS is currently considered the most accurate method for local staging of rectal cancer. The performance of ERUS in staging rectal cancer may be overestimated in the literature due to publication bias[4]. A recent analysis of 4118 subjects reported an overall mean T-staging accuracy of 85% (median, 88%) and N-staging accuracy of 75% (median, 76%). Both T-staging and N-staging accuracy rates declined over time with the lowest rates reported in more recent literature[4].

CT

The vast majority of patients with clinically localized rectal cancer have an abdominopelvic CT prior to surgical resection, in an effort to identify intra-abdominal metastatic disease prior to a curative or radical resection. However, the role of CT in the preoperative locoregional staging of rectal cancer is much more limited. In fact, the accuracy of CT for T-stage (53% to 94%) and N-stage (54% to 70%), are substantially lower overall than that of ERUS[2].

MRI

The traditional body-coil MRI is less accurate than ERUS and is rarely used for locoregional staging of rectal cancer. However, newer techniques of endorectal coil MRI and phased-array MRI have been reported to be 66% to 92% accurate in determining T-stage, and can reliably determine extent of tumor mesorectal involvement in up to 100% of cases[2,5,6]. Although promising in the preoperative staging of rectal cancer, MRI is limited by its relatively small field of view (when using the endorectal coil), expense, and patient intolerance. Similar to ERUS, restaging patients treated with preoperative chemoradiation using MRI does not appear to be as accurate. In a study of 50 patients who underwent restaging, MRI had the accuracy of 52% in T stage and 68% in N stage[7]. Most of the inaccuracy in both T and N stages was caused by overstaging, believed to be a result of the inability of MRI to differentiate treatment-induced fibrosis from viable tumors. MRI may play an important role in the future by determining preoperatively whether the mesorectal fascia has been breached by tumor, and thus optimize patient selection for neoadjuvant therapy. The results of an ongoing prospective, multicenter trial should better define the role of MRI in the preoperative staging of patients with rectal cancer.

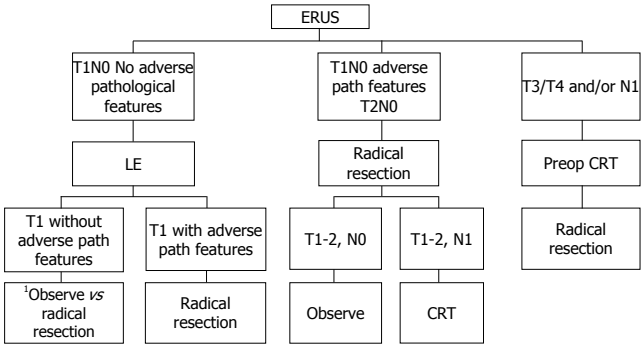


Figure 2 Treatment algorithm for patients with rectal cancer and no evidence of distant metastases. LE: local excision; CRT: chemoradiation therapy. Observation following a LE of a T1 adenocarcinoma, even with good pathological features, may result in 20% local recurrence at 10 years.

PET

PET scanning has been used in the postoperative evaluation of potential recurrences after a curative resection of rectal cancer. This is often initiated by a rising CEA level. However, preliminary data from a prospective trial conducted at our institution suggests that PET may have a role in determining locally advanced rectal cancer response to neoadjuvant chemoradiation[8]. As such, PET can potentially guide changes to the type of neoadjuvant chemoradiation to increase tumor response, as well as guide extent of subsequent surgical therapy. An ongoing, large, prospective study aims to confirm these encouraging results.

SELECTION OF CURATIVE RESECTION

Local excision is likely to be curative in patients with a primary tumor which is limited to the submucosa (T1N0M0), without high-risk features (i.e., poorly differentiated, vascular and neural invasion) and in the absence of metastatic disease. However, recent retrospective series with long-term follow-up suggest that even T1 rectal cancers without high-risk features have higher recurrence rates than expected[9-12]. Therefore, an increasing percentage of these patients are undergoing radical rectal resection. The decision to pursue a radical resection versus a local excision for an early staged rectal cancer is most difficult when the radical resection would require a permanent colostomy. Careful discussion of risks and benefits with the patient is particularly essential in this circumstance.

Once the tumor invades the muscularis propria (T2), radical rectal resection in acceptable operative candidates is recommended. In patients with transmural and/or node positive disease (T3/T4 and/or N1) with no distant metastases, preoperative chemoradiation followed by radical resection according to the principles of TME has become widely accepted (Figure 2). In patients with metastatic disease, complex and interrelated variables such as patient co-morbidities, patient expectations, and

resectability of metastases must be considered when planning surgical therapy. For patients with unresectable distant metastatic disease, surgical excision of the primary rectal cancer may still be considered when palliation of symptoms is anticipated.

CHEMORADIATION THERAPY

The use of perioperative chemoradiation therapy (CRT) for rectal cancer continues to evolve. Based largely on the results of two multicenter trials, the 1990 NIH Consensus Conference on rectal cancer recommended postoperative chemoradiation for patients with transmural and/or node positive rectal cancer[13]. Although postoperative therapy for stage II/III rectal cancer remains a reasonable option, many centers have adopted a treatment strategy of using preoperative chemoradiation therapy. The benefits of neoadjuvant chemoradiation therapy have been well documented, and include tumor regression and downstaging associated with increased tumor resectability and a higher rate of sphincter preservation[14-18]. Moreover, complete pathologic response rates up to 10% to 25% can be achieved[18-25]. The German Rectal Cancer Study Group recently completed a large, prospective, randomized trial that compared preoperative versus postoperative chemoradiation in the treatment of clinical stage II and III rectal cancer[26]. They concluded that, although there was no difference in overall survival between the two groups, there was a significant reduction both in local recurrence rate (6% *vs* 13%, $P = 0.006$) and treatment toxicity in the preoperative group. Although the quality of life for patients treated with preoperative CRT may transiently decrease, this finding is counterbalanced, in large part, by the potential for improved oncologic outcome in properly selected patients[27].

Current studies evaluating treatment outcomes in rectal cancer patients with a complete or near complete response to neoadjuvant chemoradiation have demonstrated improved survival compared to partial responders or non-responders[14,18]. In one report, patients with a complete pathological response had a 5 year disease-free survival of 95.2% compared to 55.4% for those with a partial or no pathological response ($P = 0.03$)[14]. In a recent report from MSKCC, 297 patients with locally advanced (T3-4 and/or N1) rectal cancer were treated with preoperative chemoradiation therapy followed by TME[28]. Patients who achieved > 95% pathological response from preoperative CRT had a significantly improved 10 year OS and RFS rates, when compared to those patients with a < 95% pathological response[28].

In light of the significant response rates that can be achieved with preoperative therapy, some have suggested limiting further surgical therapy to transanal excision alone[29,30]or observation[31,32] for patients with a complete response. This approach is limited by the difficulty in precisely determining tumor response to chemoradiation and assessing residual mesorectal lymph node involvement. Currently, assessment of tumor response is determined postoperatively by objective measurements of tumor volume in the surgical specimen compared to preoperative clinical staging; however, preoperative staging is based on subjective evaluation and, with current methods and technology, remains unreliable following chemoradiation. ERUS is considered the most accurate way to stage rectal cancer. But after radiation therapy, it is difficult to distinguish between residual tumor and radiation fibrosis and accuracy decreases to 47%-58%[33-37].

Similarly, restaging patients treated with preoperative chemoradiation using MRI does not appear to be accurate[7]. As discussed earlier, a preliminary report from MSKCC showed response assessment may be improved with the use of FDG-PET scanning[8]. In a series of 15 patients, FDG-PET scanning was able to more accurately assess treatment responses; confirmation with a large, prospective study is ongoing.

In patients that undergo transanal local excision, there is a risk of leaving residual disease in the mesorectum, even after combined preoperative chemoradiation therapy. Many studies have reported 1.8% to 16% of patients with lymph node involvement despite a complete pathological response in the primary tumor[38-40]. Given the inability of existing imaging modalities to reliably confirm the eradication of mesorectal nodal metastases, patients undergoing TAE alone following preoperative CRT are at risk for local failure due to residual nodal disease. Currently, there are no data to support the routine use of TAE in these patients, and definitive treatment should continue to be rectal resection with TME.

These data strongly support the need for prospective clinical trials designed to optimize the combination and sequencing of multidisciplinary neoadjuvant therapy in order to maximize survival and locoregional control rates in rectal cancer patients. If the response rate can be enhanced, it may permit less radical surgery in patients with complete responses to preoperative therapy and adjust the dose intensity or duration of postoperative chemotherapy. Ultimately, the aim of this approach is to be able to individualize or customize a patient's treatment based upon expression of molecular markers, genetic signatures using gene arrays and response to systemic therapy preoperatively.

In patients treated with preoperative chemoradiation, surgical resection is generally deferred until 6 to 8 weeks following completion of therapy in order to allow maximal tumor response, as well as patient recuperation from the toxicities sometimes associated with chemoradiation. Although the benefit of a prolonged interval from completion of chemoradiation to surgery is unclear, when clinically necessary it does not appear to increase the operative blood loss, operative time, and positive margin rate[41].

EARLY RECTAL CANCER (T1/T2 AND N0)

Local excision

Transanal local excision (LE) for T1 rectal cancer offers minimal morbidity and minimal long-term functional problems compared to radical resection (i.e., APR or LAR). On the other hand, recent evidence suggests that patients treated with LE have higher local recurrent rates than those treated with radical resection[10-12,42]. Preoperative

Table 1 Oncologic outcome for transanal excision of rectal cancer

Study (yr)	*n*	Follow-up (mo)	Local recurrence (%)	Overall survival (%)
Taylor *et al* (1998)[45]	24	52	T1: 40 T2: 50	67
Chakravarti *et al* (1999)[44]	52	52	T1: 11 T2: 62	66
Steele *et al* (1999)[43]	110[1]	48	T1: 7 T2: 20	T1: 87 T2: 85
Mellgren *et al* (2000)[42]	108	53	T1: 17 T2: 46	69
Paty *et al* (2002)[10]	125	80	T1: 17 T2: 26	T1: 74 T2: 72
Endreseth *et al* (2005)[12]	35	24-97	T1: 12	T1: 70
Madbouly *et al* (2005)[11]	52	55	T1: 29	T1: 89

[1]Patients with T2 cancers on pathology were treated with postoperative chemoradiation.

staging is relatively inaccurate and rates of LR vary widely from 7% to 40% after LE for T1 tumors[10-12,42-45]. Based on more recent, larger studies with longer follow-up, the LR rate appears to be approximately 10% to 25%. In those patients that recur after LE, only 50% or less will ultimately be cured by radical resection for salvage[46]. Because local excision does not remove the lymph node bearing tissue of the rectum (mesorectum), optimal patient selection is imperative in order to diminish the likelihood of offering this procedure to a patient whose rectal cancer might have lymph node metastases.

The risk of lymph node involvement is 0%-12% for T1 cancers, 12%-28% for T2 cancers, and 36%-79% for T3 cancers[47]. Features associated with a significantly increased risk of lymph node metastases include poor differentiation, lymphovascular invasion, and size greater than 3 centimeters[48,49]. It is therefore not surprising that, following local excision, local regional recurrence rates can be as high as 11%-29% for T1 tumors, 25%-62% for T2 tumors, and > 40% for T3 tumors[10,11,47]. Overall survival has been reported from 70% to 89% in recent series of properly selected patients (Table 1)[10-12,43,44]. However, most studies on transanal excision have follow-up data of less than 5 years and a relatively small sample size. This, in addition to the long natural history of the disease, makes conclusions on long term efficacy difficult. In 67 T1 patients treated by LE, a study at Memorial Sloan Kettering Cancer Center reported a 74% 10 year DSS[10].

In patients who develop a locoregional recurrence following local excision of rectal cancer, salvage with a radical resection is possible, with several small series reporting a 50% to 88% disease-free survival (DFS)[44,50]. After final pathology is available from a LE, consideration should be given to immediate radical resection (i.e., within 30 d). Thus, high-risk tumor characteristics and a location or size that does not enable a re-excision with clear margins warrants radical salvage surgery in order to achieve maximum local disease control. In one report of 21 patients at MSKCC, immediate radical surgery in stage I patients with adverse features was superior to those treated initially with LE followed by salvage surgery at the time of local recurrence; 94% DFS was noted in the group treated with immediate radical resection for adverse pathology versus 55% in the cohort treated with radical surgery after LR was documented[51]. A recent report from the Mayo clinic also showed that LE followed by radical surgery within 30 d does not compromise outcome compared with primary radical surgery. In the largest series published to date, 49 patients who underwent successful surgical salvage of local recurrence after LE of T1 rectal cancer, 55% required an extended pelvic dissection with en bloc resection of adjacent pelvic organs[46]. Despite the fact that 47 of 49 patients had complete resection of their pelvic disease, 58% had recurred or died of disease within 33 mo. Five year DSS was 53%[46].

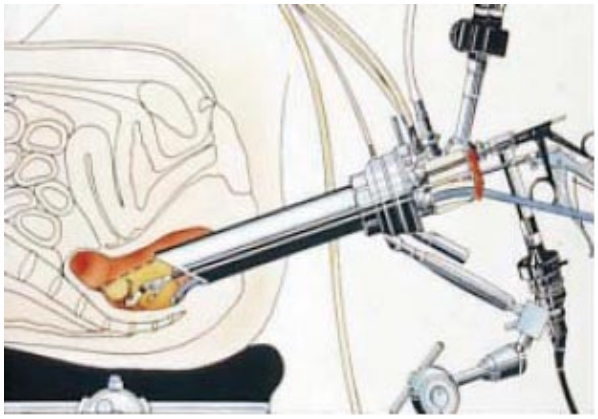


Figure 3 Specialized equipment in use for the performance of TEM[90].

Currently, local excision for cure is recommended only for carefully selected T1 tumors without high-risk features[9,10]. Patients must be followed closely and for a long period, since almost a third of local recurrences after TAE of early stage rectal cancer occur 5 years or more after local excision[10]. The role of adjuvant chemotherapy and radiation after LE is not defined at this time. Local excision is also an option for palliation in patients with locally advanced rectal cancer or stage IV patients unsuitable for radical resection[52].

Transanal endoscopic microsurgery (TEM)

TEM is an option for excising rectal cancers that are otherwise inaccessible by standard transanal excision[53]. Using a specially designed 40 millimeter diameter and 25 centimeter long operating endoscope, tumors located as high as 10 centimeters anteriorly, 15 centimeters laterally, and 20 centimeters posteriorly can be excised under direct vision (Figure 3). TEM provides a technique for full thickness excision of both benign and properly selected malignant lesions (i.e., T1 with no high risk features) that are too high for transanal excision and would otherwise require radical resection[53]. It is imperative to keep in mind that the selection criteria for TEM are the same as those for local excision (described above)[54]. When used on appropriately selected early-stage lesions, TEM can achieve oncologic results similar to those of radical resection,

Table 2 Oncologic outcome for potentially curative radical rectal resection of rectal cancer according to the principles of TME

Study (yr)	*n*	Follow-up mo	Dukes stage *n* (%)	Local recurrence (%)	Overall survival (%)
Enker *et al* (1995)[57]	246	72	B: 99 (40) C: 147 (60)	7	LN negative: 87 LN positive: 64
Heald *et al* (1998)[56]	519	99	A: 102 (20) B: 167 (32) C: 142 (27) D: 108 (21)	3	80 (DFS)
Martling *et al* (2000)[55]	381	24	A: 128 (34) B: 140 (37) C: 112 (29) Not Documented: 1 (< 1)	6	79
Wibe *et al* (2002)[58]	686	29	A: 165 (24) B: 261 (38) C: 260 (38)	7	Not Reported
Nesbakken *et al* (2003)[59]	134	38	A: 38 (28) B: 56 (42) C: 40 (30)	9	66
Bulow *et al* (2003)[64]	311	36	A 73 (23) B: 143 (46) C: 93 (30) Not documented: 2 (1)	11	77

LN: Lymph node; DFS: Disease free survival.

while limiting morbidity and mortality[54].

LOCALLY ADVANCED RECTAL CANCER (T3/T4 AND/OR N1)

During the planning and conduct of a radical operation for a locally advanced rectal cancer, a number of surgical management issues are considered, including: (1) total mesorectal excision (TME); (2) autonomic nerve preservation (ANP); (3) circumferential resection margin (CRM); (4) distal resection margin; (5) sphincter preservation and options for restoration of bowel continuity; (6) laparoscopic approaches; and (7) postoperative quality of life. The sections that follow examine each of these issues.

Total Mesorectal Excision (TME)

TME is a technique which requires precise dissection in an areolar plane between the visceral fascia that envelops the rectum and mesorectum and the parietal fascia overlying the pelvic wall structures. The end result of this procedure, when performed properly, is an intact mesorectum containing the draining lymph nodes of the rectum. This technique also facilitates pelvic autonomic nerve preservation. TME emphasizes the achievement of negative CRM and distal margins, thus optimizing the oncologic outcome for the patient. TME has been shown to achieve a negative CRM in up to 96% of resected specimens[55]. Most importantly, large series from surgical teams worldwide using TME techniques have reported local failure rates as low as 3% and overall 5-year survival of up to 80% (Table 2)[55-59]. This compares favorably

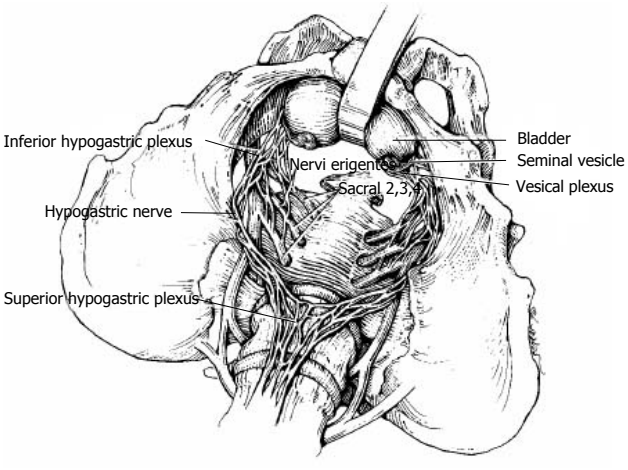


Figure 4 Diagram of pelvic autonomic nerve anatomy[73].

with large reviews of standard surgery that reported local recurrence rates of 15% to 19%, with some studies reporting local failure as high as 48%[60,61]. In fact, in studies comparing rectal resection according to the principles of TME to historical controls of standard, blunt mesorectal dissection, the patients treated with TME consistently have lower local recurrence rates[55,62-64]. Currently, TME should be considered an integral aspect of the optimal surgical management of the patient with locally advanced rectal cancer.

Autonomic nerve preservation

The sympathetic nerves of the pelvis originate from the T12 to L3 ventral nerve roots, ultimately forming the preaortic superior hypogastric plexus (Figure 4)[65]. Distal to the aortic bifurcation, the superior hypogastric plexus forms the hypogastric nerve, which may be intimately associated with the visceral fascia of the mesorectum. Injury to the hypogastric sympathetic nerve trunks results in increased bladder tone with reduced bladder capacity, voiding difficulty, impaired ejaculation in men, and loss of vaginal lubrication and dyspareunia in women. The parasympathetic nerves of the pelvis (nervi erigentes), arising from the S2 to S4 ventral nerve roots, join the hypogastric nerves (sympathetic) on the pelvic sidewall to form the inferior hypogastric plexus (pelvic autonomic nerve plexus) (Figure 4)[65]. Damage to the parasympathetic nerves leads to erectile dysfunction, impaired vaginal lubrication, and voiding difficulty.

Truncal ANP is defined as preservation of the anterior nerve roots of S2, S3, and S4, the superior hypogastric nerves, and the pelvic autonomic nerve plexus[57]. With careful autonomic nerve preservation, postoperative genitourinary and sexual dysfunction can be reduced from 25% to 75% to as low as 10% to 28%[66]. More specifically, neurogenic bladder can be reduced from 9% to 40% with conventional rectal resection to as low as 0% to 11% with TME and ANP[66-68]. The rate of sexual dysfunction may be further reduced by using intraoperative nerve stimulators to help identify and preserve the pelvic autonomic nerves[69]. It must be emphasized, however, that



Figure 5 MRI of rectal cancer with demonstration of circumferential resection margin (CRM)[6]; Black arrows: rectal cancer; White arrows: mesorectal fascia; Dashed line: CRM, which is defined as the shortest distance from rectal cancer to the lateral resection margin of the mesorectum.

factors other than ANP, such as history of chemoradiation, patient co-morbidities (i.e. -atherosclerosis, diabetes mellitus, hypertension), medications (i.e. beta-blockers), and alcohol use may contribute to genitourinary and sexual dysfunction following radical rectal resection.

Circumferential resection margin (CRM)

The importance of the CRM in minimizing local recurrence of rectal cancer was first reported in 1986 (Figure 5). A recent series of 686 patients with rectal cancer treated with TME after a median follow-up of 29 mo documented a 5% local recurrence rate for patients with CRM > 1 millimeter and a 20% local recurrence rate for a CRM $\leqslant$ 1 millimeter[58]. Obtaining a negative CRM is likely to result in decreased rates of local recurrence, distant metastases, and death. In order to provide an optimal oncologic outcome, the surgeon must make all efforts to obtain a negative CRM, including en bloc resection of contiguous structures.

Distal resection margin

Distal spread greater than 1 centimeter beyond the mucosal edge of rectal cancer has been documented in only 10% of cases, all in poorly differentiated, node-positive lesions[70]. Recent data support this finding, suggesting that margins as small as 1 centimeter may provide acceptable oncologic results. In a series from our institution, the recurrence-free survival and local recurrence rates with 3 years of follow-up after preoperative combined modality therapy (CMT) and TME-based resection were not significantly different in patients when margins less than or equal to 1 centimeter were compared to those greater than 1 centimeter. Thus, although we advocate striving for a 2-centimeter distal resection margin when feasible, acceptable oncologic results may be achieved with margins of at least 1 centimeter, especially when resection follows CRT.

Sphincter preservation and restorative options

In patients with acceptable preoperative anorectal function, ideal body habitus and pelvic anatomy, sphincter preservation is usually possible for rectal cancer located greater than one centimeter above the upper portion of the anorectal ring. Generally, slender patients with a wide pelvis are more appropriate for sphincter preserving resection of distal rectal cancer than obese patients with a narrow pelvis[66,73]. Male patients with a long, narrow pelvis and/or enlarged prostate present a technical challenge that may preclude a restorative procedure[73]. Finally, patients with impaired preoperative anorectal function may be better treated with radical resection and permanent colostomy, thus avoiding substantial postoperative perineal morbidity[73]. Hence, it is imperative that the surgeon exercise sound clinical judgment when selecting patients for restorative rectal resection.

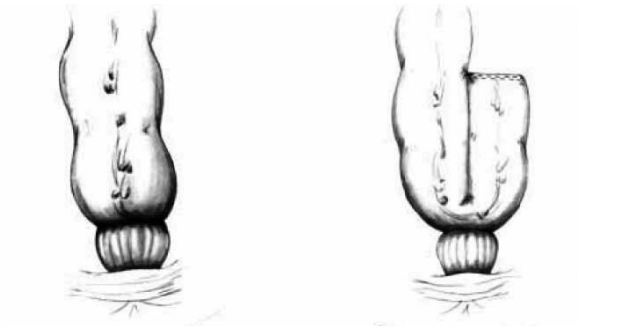


Figure 6 Illustrated comparison of a straight coloanal anastomosis and a coloanal anastomosis with a colonic J-pouch[74].

Classically, bowel continuity following low anterior resection (LAR) was restored with a straight colorectal or coloanal anastomosis. In 1986, J-pouch coloanal anastomosis was developed in order to increase colonic reservoir function and improve quality of life following an LAR that required coloanal anastomosis for restoration of bowel continuity (Figure 6)[74]. A prospective, randomized trial comparing patients with a straight CAA and a coloanal J-pouch anastomosis using the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 and QLQ C-38 demonstrated an improved postoperative quality of life in the patients reconstructed with a J-pouch[74]. Randomized studies have demonstrated the superiority of a 6 to 8 cm coloanal J-pouch anastomosis relative to a straight coloanal anastomosis, particularly during the first year following surgery[75]. There is a significant reduction in the postoperative anastomotic leak rate, number of stools per day, and improved quality of life in patients with a J-pouch versus those with a straight coloanal anastomosis after LAR[76].

One disadvantage of the colonic J-pouch is that up to 25% of patients treated with an LAR are not candidates for the procedure, due in large part to the somewhat bulky size of the pouch[77]. In 1997, an alternative procedure, known as the transverse coloplasty, was introduced in an attempt to create a distal colonic reservoir (Figure 7)[78]. A recent randomized trial demonstrated comparable functional results, with improved neorectal sensitivity, when patients undergoing transverse coloplasty-anal anastomoses were compared to those reconstructed with a J-pouch[77]. However, another trial documented

increased leak rates with transverse coloplasty and no differences in bowel function, when compared to a colonic J-pouch[79]. Therefore, colonic J-pouch provides optimal postoperative bowel function with lower morbidity than transverse coloplasty and in most cases should be the primary method of bowel reconstruction when a coloanal anastomosis is required following an LAR[74,79]. However, when reconstruction with a J-pouch is not technically feasible, transverse coloplasty-anal anastomosis provides a reasonable option for bowel reconstruction.

Laparoscopic approaches

Data from small, non-randomized studies evaluating laparoscopic-assisted rectal cancer resection suggest that laparoscopic-assisted TME is feasible when performed by experienced surgeons[80]. From these non-randomized reports, oncologic outcome does not appear to be impaired by laparoscopic rectal cancer resection[81-83]. In addition, short-term morbidity may be reduced in the laparoscopic group, while oncologic outcome is not compromised[81-83]. However, pending prospective, randomized trials focusing on laparoscopic resection of rectal cancer need to be concluded before definitive recommendations can be made concerning the safety and oncologic efficacy of these procedures.

Quality of life following radical resection

Although improved outcome is the ultimate goal for the surgical treatment of rectal cancer, there has recently been increased interest in the quality of life of patients following radical rectal resection. As previously discussed, performing a rectal resection according to the principals of TME with ANP substantially reduces the incidence of postoperative genitourinary and sexual dysfunction. In fact, a recent series reported that even in the face of postoperative fecal incontinence, genitourinary dysfunction, and sexual dysfunction, patients were satisfied with their quality of life following rectal resection[84]. A recent 4-year prospective study of 329 patients with rectal cancer reported the quality of life following radical resection[85]. Using the EORTC QLQ-30 and CR-38 questionnaires, they report that patients undergoing LAR have improved quality of life when compared to patients undergoing APR. In addition, patients who had no stoma, or had their stoma reversed, reported a substantially improved postoperative quality of life compared to patients with a permanent stoma[85].

Another large series with 2 years of follow-up, using the EORTC QLQ-C30 and QLQCR-38 questionnaire, reported opposite results. Patients with a permanent stoma reported significantly better social function ($P = 0.005$), less anxiety ($P = 0.008$), and higher self-esteem ($P = 0.0002$) than patients who underwent restoration of bowel continuity[86]. These findings have been supported by others in the literature[87]. In addition, others have reported that postoperative quality of life improves with time, and should therefore be evaluated in a dynamic fashion[88]. To add further complexity to this issue, patients who are treated with a very low colorectal or coloanal anastomosis may have a decreased postoperative quality of life than patients treated with APR and permanent stoma[89]. It is clear that postoperative quality of life is dependent upon the interaction of patient factors (i.e. co-morbidities and preoperative anorectal function), tumor factors (i.e. extent of local invasion, distance from the anal verge), and surgical factors (i.e. level of the anastomosis). However, the conflicting data in the literature concerning quality of life evaluation for patients with resected rectal cancer underscore the importance of the development of more sensitive, validated instruments.

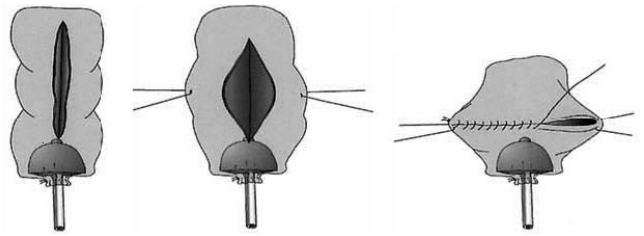


Figure 7 Technique of construction of a stapled coloanal anastomosis with a transverse coloplasty pouch. Alternatively, the pouch may be hand-sewn to the anal canal[77].

In conclusion, surgery for rectal cancer continues to develop towards the ultimate goals of improving local control and overall survival, maintaining quality of life, and preserving sphincter, genitourinary, and sexual function. In appropriate patients, minimally invasive procedures, such as local excision, TEM, and laparoscopic resection allow for improved patient comfort, shorter hospital stays, and earlier return to preoperative activity level. Currently, local excision for cure is recommended only for carefully selected T1 tumors without high-risk features. Recent studies suggest that in patients with resectable rectal cancer, a response to preoperative chemoradiation is predictive of decreased local recurrence and improved survival. As response rates to neoadjuvant therapy continue to improve, it will enable more patients to undergo sphincter-sparing surgery, and will additionally provide a guide to postoperative chemotherapy regimens. However, at this time the existing imaging modalities are limited by their inability to confirm eradication of mesorectal nodal metastases; thus, patients undergoing TAE alone following preoperative chemoradiation therapy are at risk for local failure due to unidentified residual nodal disease. Currently, there are no data to support the routine use of TAE in these patients, and definitive treatment should continue to be rectal resection with TME. By strictly adhering to the principles of TME with autonomic nerve preservation, maintenance of urinary and sexual function can be achieved in the majority of patients undergoing a curative radical rectal cancer resection. The ultimate goal is for physicians to be able to individualize a patient's treatment based upon expression of molecular markers, genetic signatures using gene arrays and response to systemic therapy preoperatively, optimizing the combination and sequencing of multidisciplinary neoadjuvant therapy in order to maximize survival and locoregional control rates.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Update on obesity surgery

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Abstract

The prevalence of obesity in the United States has reached epidemic proportions. With more than 30 million Americans clinically obese, the younger population has also been affected. Surgical therapy should be offered to the severely obese patient who is refractory to nonsurgical therapy, as established by the 1991 NIH Consensus Conference on Gastrointestinal Surgery for Severe Obesity. Surgery is currently the most effective therapy for weight loss. It is far more effective than any other treatment modality, both in terms of the amount of weight loss and in terms of durability in maintaining weight loss.

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Key words: Obesity; Gastrointestinal surgery; Roux-Y gastric bypass

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INTRODUCTION

The prevalence of obesity in the United States has reached epidemic proportions[1]. With more than 30 million Americans clinically obese, the younger population has also been affected. The number of obese young adults between the ages of 18 and 29 has increased by nearly 50% in the 1990's[2-4], and currently, nearly 25% of children are believed to be obese[5]. Obesity now represents a staggering 5% of the total health care costs in the United States[6]. This, however, is not only an American problem. The prevalence of obesity worldwide is also increasing. In England, the number of obese men and women has tripled over the twenty years between 1980 and 2000[7]. More than 1.5 billion people worldwide are thought to be obese or overweight[8]. Obesity, clinically defined as a body mass index (BMI) greater than 30 kg/m^2, is associated with comorbid conditions affecting nearly every organ system, including the cardiovascular, endocrine, renal, pulmonary, gastrointestinal, and musculoskeletal systems. Type 2 diabetes, hypertension, hyperlipidemia, obstructive sleep apnea, stroke, heart disease, renal disease, degenerative joint disease, venous disease and several forms of cancer have all been associated with obesity[9,10]. In addition, the obese are frequently afflicted by depression, anxiety and low self-esteem[11]. In the United States, obesity leads to a significant decrease in life expectancy and an annual cost of approximately $100 billion, including nearly $4 billion in lost productivity[12,13]. In severely obese individuals (BMI> 35 kg/m^2), the mortality is 12 times higher in men aged 25-34 years and 6 times higher in men aged 35-44 years compared to age-matched nonobese control subjects[14].

In severely obese people, diet, exercise, and pharmacotherapy can produce a modest weight loss in the range of 2%-10% at 1 year. Unfortunately weight regain is common. The worsening obesity crisis, combined with the lack of a durable medical treatment option, has led to an increased interest in surgical approaches for the treatment of obesity. Bariatric surgery may be broadly defined as surgical treatment strategies designed to limit caloric intake, decrease nutrient absorption, or both. Procedures designed to limit caloric intake, termed restrictive procedures, include gastric banding or the placement of intragastric balloons. Surgical procedures that decrease nutrient absorption are termed malabsorptive procedures. Jejunal-ileal bypass, a now historical operation, can be considered a pure malabsorpitve procedure. The most commonly performed bariatric operations, such as gastric bypass and biliopancreatic diversion, introduce elements of caloric restriction and partial nutrient malabsorption[15]. Worldwide, the gastric bypass is the single most frequently performed operation for obesity[16], with more than half being done laparoscopically[17]. Most importantly, patients who undergo gastric bypass have been able to achieve long-term excess weight loss in the range of 49%-62%[18,19,20].

For severely obese adults with weight-related comorbidities, surgery provides the most effective and durable approach for clinically significant weight loss. There is yet no consensus about surgery for severely obese adolescents, but it is nonetheless gaining in popularity, with demonstration of significant and durable weight loss in

individuals 12 to 18 years old[5].

INITIAL APPROACH TO THE OBESE PATIENT

Overweight and obesity are independent risk factors for increased mortality. Dramatic increase in mortality is noted when BMI exceeds 30 kg/m^2. This risk further increases the longer the duration of obesity[23,24]. Disappointingly, only 27% to 42% of obese patients are advised by their healthcare professional to lose weight[21,22]. A variety of non-surgical options are available for the treatment of obesity, including exercise therapy, behavioral and dietary modification, and pharmacotherapy[25]. Successful long-term weight loss may be defined as maintaining a 10% weight-loss over a period of one year, or more strictly as an average weight loss of 30 kg for an average of 5.5 years[26]. For the majority of obese individuals, sufficient weight loss is infrequently obtained by conventional methods.

Dietary modification and nutritional counseling is an essential first step in the treatment of obesity. Unfortunately, no dietary approach has ever demonstrated sustained weight reduction in the severely obese population[6]. Recently, a randomized trial compared common diets for efficacy of weight loss[28]. The Atkins, Ornish, Weight Watchers and Zone diets were compared in patients with a mean BMI of 35 kg/m^2. None of the diets studied produced a mean weight loss of more than 10 kg at one year. Behavioral therapy in combination with dietary modification appears to be more effective than either method alone, but most patients trend towards their pre-treatment weight within 5 years of treatment[29]. Physical activity, as a method for weight reduction, is a necessary component of most weight-loss programs. Consistent exercise has been shown to effect longer lasting weight loss, and the level of physical activity directly correlates with the degree of weight loss[30]. Diet with exercise produces superior results compared to either diet or exercise alone. At one-year follow-up, the addition of exercise to diet alone appears to give more stable weight loss. However, the total weight loss still remains modest, in the range of 6-9 kg after 1 year[31].

Appetite suppressants are also used to effect weight loss. These include serotonin selective re-uptake inhibitors (SSRI's), serotonergic, and noradrenergic agents. Such anorectic agents are usually used in combination with a designed low-calorie diet. With one exception, all studies evaluating their efficacy have shown that a combination of diet and appetite suppressants appear to be effective during the first 6 mo of treatment, but fail to show long-term effectiveness beyond 12 mo[25,32]. Orlistat, a newer agent that induces fat malabsorption, similarly will only lead to an initial excess weight loss of 10%[6]. Long-term studies are less optimistic. After 52 wk of three times per day therapy, when combined with a low calorie diet, orlistat will only lead to a weight loss of 5%[27].

OBESITY SURGERY

Surgical therapy should be offered to the severely obese patient that is refractory to nonsurgical therapy, as established by the 1991 NIH Consesus Conference on Gastrointestinal Surgery for Severe Obesity[33]. The impetus for the introduction of surgical therapy for obesity was borne from the high incidence of long-term weight regain in the severely obese. Patients are eligible for bariatric surgery if they have failed nonsurgical treatments and have a BMI > 40 kg/m^2 or BMI > 35 kg/m^2 with comorbidities.

Preoperative considerations

The obese patient undergoing abdominal surgery warrants special consideration in the preoperative workup. These patients have an overall higher mortality compared with nonobese patients when undergoing gastrointestinal surgery (6.6% *vs* 2.6%)[34]. As they carry higher risks for cardiovascular disease, pulmonary and thromboembolic complications, and wound infections, special care must be taken in preparing these patients for surgery[35]. Operative weight has been shown to directly correlate with an increase in cardiovascular events in both men and women[36]. Risk stratification and appropriate preoperative intervention, therefore, is important in this patient population. Low-risk patients should receive perioperative beta-blockade, while patients that are intermediate and high-risk should undergo noninvasive cardiac testing preoperatively[35].

The risk for deep venous thrombosis and pulmonary embolism is also higher in the obese population compared with the general population. Specific comorbidities in these patients have been identified that increase the risk of deep venous thrombosis (DVT) or pulmonary embolism (PE). These include the presence of venous stasis disease, BMI > 60 kg/m^2, truncal obesity, and obstructive sleep apnea[37]. The presence of clinically severe obesity alone is associated with a high rate of pulmonary embolism in postoperative autopsies[38]. The preoperative use of 5000 units of subcutaneous unfractionated heparin, and every 8 h thereafter during the hospitalization, in conjunction with sequential compression devices, has been shown to provide adequate DVT prophylaxis[39]. Alternatively, low-molecular weight heparin may be used for DVT prophylaxis[40,35]. In patients with a very high risk for postoperative DVT/PE, the preoperative placement of an inferior vena cava (IVC) filter should be considered[41].

Jejunoileal bypass

The field of obesity surgery began in the early 1950's with the jejunoileal bypass, a malabsorptive procedure in the strictest of definitions[42]. As its name implies, this procedure bypasses a large segment of small intestine by connecting the proximal jejunum (roughly 14 inches from the ligament of Treitz) to distal ileum, about 4 inches proximal to the ileocecal valve (Figure 1A). Weight loss is produced by malabsorption of macronutrients, namely carbohydrates, fats, and proteins[17]. Severe diarrhea and nutritional deficiencies frequently result, leading to significant metabolic abnormalities, nephrolithiasis, cholelithiasis, osteopenia, and hepatic cirrhosis. In the past, metabolic bone disease and vitamin deficiencies often necessitated reversal of the procedure[43]. With time, other procedures carried a significantly lower complication

profile and thus supplanted the jejunoileal bypass[44]. This procedure is now of historical significance only.

Biliopancreatic diversion

The vast majority of complications that resulted from jejunoileal bypass resulted from the very long "blind" limb of intestine that neither biliopancreatic juices nor victuals flowed through. Newer malabsorptive procedures have eliminated this "blind" limb[18]. The biliopancreatic diversion (BPD), first described by Scopinaro in 1979[45], involves the creation of a distal gastrectomy with a Roux-Y reconstruction using a 250-cm alimentary tract limb as measured from the ileocecal junction (Figure 1B). The long biliopancreatic limb is anastomosed to the distal ileum, creating a short (50-cm) common channel for nutrient absorption[47]. Weight loss occurs because the ingested food is not entirely digested and absorbed. This is a true malabsorptive procedure. In contrast to purely restrictive procedures, BPD allows patients to continue eating "normally" while achieving significant weight loss[54]. In BPD, nutrient absorption occurs predominantly in the short distal common channel, where both the chyme and biliopancreatic juices mix.

Short-term weight-loss has been reported at almost 15% at 4 mo[47] and more importantly, in a published series of more than 2000 patients[46], a mean long-term weight reduction of 75% of the initial excess weight was achieved. Late complications include anemia, stomal ulcers, bone demineralization and neurologic dysfunction due to vitamin deficiencies. The vitamins most affected are the fat-soluble vitamins A, D, K, and E. Calcium and iron, primarily absorbed in the duodenum, can also be significantly affected. Protein calorie malnutrition has been documented, as well as hepatic failure and polyarthritis[48,49]. Due to the late onset of significant metabolic complications associated with the procedure, it has fallen out of favor in the United States[48].

Duodenal switch

The biliopancreatic diversion with duodenal switch (BPD-DS) adds a sleeve gastrectomy (instead of distal gastrectomy) and duodenoileostomy to the BPD (Figure 1C). The common channel is increased in length from 50 to 100 cm. This procedure was first performed in 1990, with several modifications in the duodenal switch portion to its current form[54]. BPD-DS was first performed laparoscopically by Gagner *et al* in 1999[53]. The duodenal switch modification was made to try to reduce the complication rate, while maintaining reliable weight loss. Although durable weight loss is comparable to the BPD, long-term complications, mostly intractable diarrhea, are still a major concern[50]. Micronutient supplementation is still mandatory to help minimize the risk of vitamin deficiency syndromes and osteoporosis[54]. Nonetheless it remains a very effective treatment for the superobese patients (BMI > 50 kg/m^2). Excess weight loss ranges from nearly 60% to greater than 90% in 10 to 24 mo[51]. At 8 years, excess weight loss may be as high as 70%[52]. In experienced hands, this procedure can be performed safely via the laparoscopic approach[53].

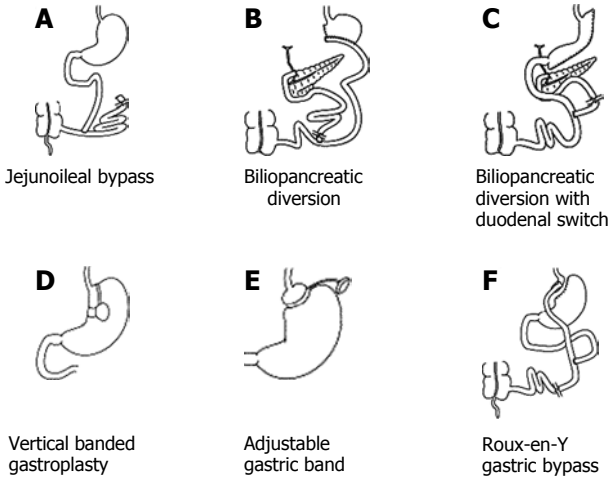


Figure 1 Diagrammatic representation and comparisons of weight loss operations, past and present.

Vertical banded gastroplasty

The vertical banded gastroplasty (VBG) was first described by Mason in 1982[55]. This procedure is a purely restrictive procedure; it leaves the rest of the gastrointestinal tract intact for nutrient absorption. In this procedure, a full thickness hole is made with a circular stapling device in the proximal stomach. Using this hole, the proximal stomach is stapled, parallel to the lesser curve, up to the angle of His. This creates a pouch of approximately 50 mL capacity (Figure 1D). A band of silastic or polypropylene mesh is commonly placed through the hole, circumferentially around the distal portion of the tubularized pouch, thereby limiting the stomal outlet diameter to roughly 1 cm[48,55].

Vertical banded gastroplasty may be performed open or with laparoscopic techniques. Results of VBG vary. Excess weight loss in the first year after surgery is in the range of 60%. Patient satisfaction, meanwhile, ranges from 75% to greater than 90%[56,57]. Five-year follow-up suggests less satisfactory weight loss, with the average excess weight loss being 30%-54%. Frequently, patients cited difficulties in tolerating a regular diet[59-61]. Most disappointingly, a prospective study following patients for more than 10 years after vertical banded gastroplasty found that only 20% of patients maintained the loss of at least 50% of excess body weight[58]. Additionally, a significant portion of patients had persistent vomiting, gastroesophageal reflux, or heartburn. Furthermore, the rate of re-operation may be as high as 29%[62]. When compared to gastric bypass, multiple studies have shown the superior results of gastric bypass over VBG in terms of durable weight loss and long-term outcome. Subsequently, VBG has fallen into disfavor[43,63,64].

Gastric banding

Adjustable gastric banding is a restrictive procedure that does not involve stapling of the gastrointestinal tract. It is considered the least invasive of the operations for severe obesity[65]. The key feature of the current gastric banding devices is the adjustability of the outer diameter of the gastric band. Thus, oral intake can be restricted if the band is tightened, or the band can be loosened in patients that

present with signs or symptoms of obstruction. Dietary compliance is mandatory for the success of the adjustable gastric band. Patients can bypass the effects of the banded gastric pouch by consuming high-calorie liquid diets, thus emphasizing the need for continued cooperation postoperatively between the physician and patient.

Initially described as an open procedure by Kuzmak in 1986, an adjustable silicon band is used to encircle the proximal stomach, creating a small gastric pouch and narrowed outlet[66]. The only device currently approved in the United States is the Lap-Band (Inamed Health, Santa Barbara, California), but is one of several commercially available devices worldwide. The Lap-Band consists of a silicon adjustable elastic band that is placed around the proximal stomach via the pars flaccida, creating a pouch volume of 10-15 mL (Figure 1E). A subcutaneous port enables percutaneous needle access to adjust the volume of the gastric band and consequently the size of the gastric pouch outlet[67]. Although results in Europe have been encouraging[68], the early results in the United States were considered poor relative to other bariatric procedures, with only 36% excess weight loss after three years. Later studies showed improvement with average excess weight loss of 42% at one-year follow-up[69]. A more recent prospective study produced much more impressive results[70] with mean excess weight loss of 50% at one year, 61% at two years, and 65% at three years.

Early complications are uncommon in this laparoscopic procedure and conversion to open operation is infrequent[70,71]. Overall complication rates vary in the literature between 9%-20%[69,70], including band slippage or prolapse, dysphagia, port migration, port-site infection, tube breakage, dilatation of the gastric pouch, gastric perforation, and erosion of the band through the stomach wall[43,70-73]. Some of these complications are treated electively with laparoscopic reposition or replacement of the Lap-Band device, or with repositioning or repair of tubing or the port. Complication rates and frequency of reoperation likely reflect overall experience with the procedure and optimal band positioning. The longer European experience reflects a learning curve with decreasing morbidity and need for reoperation. Total complication rates are 9%-11% in most European studies, but 10%-20% in U.S. studies. Depending on the institution, the reoperation rate varies from 1% to 13%[43,69,70,73-75]. Malnutrition and vitamin deficiencies are uncommon.

The Swedish version of the gastric band, the Swedish Adjustable Gastric Band (AB Obtech, Sweden), was introduced in 1985 by Hallberg *et al*[76]. It consists of an inflatable double-silicon band reinforced by a Dacron net. The silicon balloon on the inner surface can be regulated by a subcutaneous port. Its complication profile is similar to the Lap Band, including band erosion, erosive esophagitis, pouch dilatation, disconnection of tubing, invagination of distal gastric wall, and leakage from the balloon[76,77]. Several studies showed promising results for the Swedish Band. At a five-year follow-up mean excess weight loss was 57%, with approximately 83% of patients obtaining more than 50% excess weight loss[78]. Similar results were obtained by Australian investigators[79] with a mean excess weight loss of 60% at 12 mo, and 69% of patients had a greater than 50% excess weight loss over the same follow-up.

A Swiss prospective, randomized trial compared 90 patients treated with Lap Band and 90 patients treated with the Swedish Adjustable Gastric Band (SAGB)[80]. The SAGB appeared more susceptible to infection and early complication. Although the patients with Lap Band lost more excess weight sooner, the two cohorts trended towards equal excess weight loss by 30 mo post-operatively. At three-year follow-up, only about half of each treatment group lost at least 50% of excess weight. An earlier study with a short follow-up of less than 12 mo showed no difference between the two gastric bands in terms of weight loss and complications[81]. An additional meta-analysis reviewing the two laparoscopic gastric bands revealed similar excess weight loss, although the Lap Band seemed to have a slightly higher long-term complication rate[82]. Overall there appears to be little major difference between the two banding systems.

Intragastric balloon

Another, much less common technique, is the placement of an intragastric balloon. First introduced in the 1980's, preliminary studies in pigs showed ambiguous results when an inflatable balloon was introduced into the stomach in an attempt to reduce stomach capacity and oral intake[15,83]. In humans, the intragastric balloon is inserted orally using a special application sheath. It is placed 10 cm distal to the gastroesophageal junction and inflated with approximately 500 mL of saline, attaining a diameter of 10 cm. The volume can be subsequently adjusted to 400-800 mL. Smaller, 200 mL, air-filled balloons are also available. Large hiatal hernias and peptic ulcer disease usually preclude the use of the intragastric balloon. Immediate complications include nausea, vomiting, and abdominal cramps, suggesting intolerance of the balloon[84]. Other complications include gastroesophageal reflux, belching, and esophageal erosions. As of yet, there are no long-term studies to prove the efficacy of this technique. Relative to other bariatric procedures, weight loss results are modest at best. The greatest reported results are in the range of 10% weight loss sustained at two years[84].

Gastric pacing

Gastric pacing, first introduced in humans in 1996[85], is not widely accepted and still considered experimental. It attempts to provide a non-malabsorptive and non-restrictive method for significant, durable weight loss. The purported mechanism of action is to disrupt normal gastric myoelectrical activity, which subsequently affects both gastric emptying and gastric relaxation. The implantable gastric stimulator (IGS; Transcend, Transneuronix, Inc., New Jersey) includes a battery-operated generator and a bipolar lead. Electrodes are placed into the muscle layer on the lesser curvature of the anterior gastric wall[86]. The electrode delivers a bipolar pulse through its connection to a pacer that sits in a subcutaneous pocket[42]. It is postulated that electrical stimulation disrupts eating behavior by causing a change in parasympathetic activity in the stomach, leading to gastroparesis[42,87]. Weight loss should therefore be induced by consistent and predictable

early satiety. Early human studies in a small cohort showed weight loss in only half the patients[85]. Later studies, also in a small number of patients, were able to demonstrate a modest, but stable weight loss[86]. Thus far, studies on gastric pacing have been plagued by mechanical problems relating to the electrical leads.

The procedure is safely and preferably performed laparoscopically[88]. A large multicenter, randomized, controlled, double-blinded American trial is under way[89]. Early results showed a mean weight loss of 11% at one year. A second round, pilot trial is in progress, employing four electrodes and thus far, a mean excess weight loss of 21% was seen at 7-mo follow-up[89]. Although these results are modest compared to other bariatric procedures, the optimal use of the electrodes, the number of electrodes, and the direction of stimulation has yet to be determined.

Gastric bypass

The Roux-Y gastric bypass, a mixed restrictive and malabsorptive procedure, is considered the gold standard for bariatric surgery[6], and is currently the procedure of choice in the United States[16]. The gastric bypass operation was first introduced by Mason *et al*[90] in 1967. In the initial description of the operation, a loop gastrojejunostomy was created. The Roux-Y gastric bypass (RYGB) was subsequently introduced in 1977[44]. In this procedure, a small gastric pouch of 15-30 mL is created by transecting the proximal stomach. Intestinal continuity is reestablished *via* a Roux-Y gastrojejunostomy (Figure 1F). With the resultant anatomy, dietary foodstuff bypasses the distal stomach, duodenum, and proximal jejunum. Unlike the true malabsorptive procedures, protein calorie malnutrition is rare. The only malabsorption that takes place with RYGB is micronutrient malabsorption, mainly folate, calcium, iron and vitamin B12.

Gastric bypass is effective for many reasons. First, the small proximal gastric pouch restricts the food portions that the patient is able to ingest. It forces the patient into good eating behaviors such as thoroughly chewing one's food and eating slowly. For the first several months after surgery, daily caloric intake ranges from 1674 to 3347 kJ. The second, equally important, contributor to weight loss is that the low level of caloric intake is well tolerated by patients; they do not usually feel hungry. Unlike most other bariatric procedures, gastric bypass results in a decrease in serum ghrelin levels. Ghrelin is a hormone responsible for satiety, and is produced by cells located in the gastric fundus and proximal duodenum in response to an empty stomach. When ghrelin is released into the bloodstream, it stimulates feeding behaviors. The level of ghrelin falls shortly after eating and is clearly one hormone responsible for satiety. After gastric bypass, ghrelin levels are low and remain low for an indefinite period of time, resulting in a prolonged decrease in appetite[91].

When open Roux-Y gastric bypass is compared to open vertical banded gastroplasty (VBG), patients who undergo gastric bypass have superior weight loss as well as long-term weight-loss maintenance[64,92]. Similarly, in a randomized trial of 83 patients, laparoscopic Roux-Y gastric bypass out-performed laparoscopic VBG[63]. Despite the relative simplicity of performing a VBG and, consequently, shorter operative times, weight reduction was significantly greater after laparoscopic gastric bypass at one and two years postoperatively. After gastric bypass, weight loss is significant and durable. Multiple studies support the use of the Roux-Y gastric bypass for the treatment of the severely obese. A mean of greater than 60% excess weight loss is seen at 1-, 2-, 3- and 5-years postoperatively[64,93-95]. Long-term follow-up has been reported at fourteen years, with an excess weight loss of 49%[96]. The best results, obtained by Fobi *et al*, show 80% excess weight loss at two years[97].

The Roux-Y gastric bypass, first described as a laparoscopic procedure in 1994 by Wittgrove *et al*[98], has proven to be safe and effective and has quickly gained popularity[99]. The operation is more challenging than the open gastric bypass, however, and a learning curve of approximately 100 cases should be expected[100,101]. Despite slightly longer operative times in the laparoscopic operation, patients who undergo laparoscopic RYGB have less blood loss, shortened hospitalization time, and a faster return to normal activities when compared to patients who undergo open RYGB. For laparoscopic RYGB, the average length of postoperative stay is 2 d[102-104]. A case series of 400 patients undergoing laparoscopic Roux-Y gastric bypass revealed an average excess weight loss of 69% after one year. This varied by BMI, with slightly diminished results in the heavier patients[99]. The study also showed importantly that operative times decreased steadily with operative experience. This finding is supported by other studies as well[101]. Wittgrove and Clark showed an excess weight loss of 80% after the first year[105]. Schauer *et al*[103] found an excess weight loss of 83% at two years and 77% at 30 mo using the laparoscopic approach.

Laparoscopic gastric bypass is also safe and effective in the super obese (BMI > 60 kg/m^2). For patients with a BMI less than 60 kg/m^2 the excess weight loss is greater than 60% at 1 year in multiple studies whereas in the super-obese, excess weight loss in the range of 50%. The complication profile, however, is similar through a range of BMIs[99,106,107]. Recently, laparoscopic Roux-Y gastric bypass was shown to be safe and effective in patients with a BMI > 70 kg/m^2[108]. Overall, the complication rate following gastric bypass is 10%-15% and mortality is approximately 0.3%[43,101]. Complications include anastomotic leak, thromboembolic events, bleeding, anastomotic stricture, hernia, marginal ulceration, vitamin malnutrition, gallstones, and wound infection. In the laparoscopic approach, complication rates sharply decrease along the surgeon's progress on the operative learning curve[101]. Wound infection and incisional hernia, the most common complication in the open operation, are found at a rate of < 5% and < 1%, respectively, in laparoscopic Roux-Y gastric bypass. Late anastomotic stricture, however, was found more commonly with the laparoscopic approach, nearing 10% in some series[101-103,109]. Nutrient and vitamin deficiencies created by this procedure are easily correctable and, more importantly, preventable with appropriate supplementation. Due to the outstanding weight loss that can be achieved with a relatively low complication profile, laparoscopic Roux-Y gastric bypass has become the bariatric procedure of choice.

COMORBIDITY RESOLUTION

Bariatric procedures in general, and the gastric bypass in particular, produce dramatic improvements of comorbidities associated with severe obesity, with documented resolution of type 2 diabetes and glucose intolerance. Although the exact mechanism is unknown, it appears that the gastrointestinal bypass procedure itself, not just the actual weight loss, plays the major physiologic role in these findings[110]. In addition, improvement or resolution was seen postoperatively in patients with other components of the metabolic syndrome such as hypertension and dyslipidemia. In a comparison of 114 case series, 64%-100% of postoperative patients showed improvement or resolution of their diabetes. A study of 11 reports showed an improvement or resolution of dyslipidemia in 60%-100% of patients. In 19 studies, hypertension showed an improvement or resolution in 25%-100% of patients[17]. Two years after obesity surgery, the incidence of hypertension, diabetes, and lipid abnormalities are reduced relative to medically treated patients. Followed to eight years, the incidence of diabetes showed the most durable reduction, while the reduction in dyslipidemia and hypertension was more modest[112,113]. With appropriate weight reduction, dramatic improvements in sleep apnea are also seen[17,111].

In conclusion, surgery is currently the most effective therapy for weight loss. It is far more effective than any other treatment modality, both in terms of the amount of weight loss and in terms of durability in maintaining weight loss. Additionally, the surgical treatment of obesity produces results that greatly improve or reverse the comorbid conditions often associated with severe obesity. These include diabetes, hypertension, dyslipidemia, and obstructive sleep apnea. This is most apparent in patients who had undergone a Roux-Y gastric bypass. The search for a less invasive, equally effective weight loss modality is still underway. Ideally a non-operative medical therapy will emerge that will produce equal or near-equal results. Until then, surgery will remain the treatment of choice for this population of patients.

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TOPIC HIGHLIGHT

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Pediatric inflammatory bowel disease

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Abstract

Inflammatory bowel disease is an important cause of gastrointestinal pathology in children and adolescents. The incidence of pediatric inflammatory bowel disease is increasing; therefore, it is important for the clinician to be aware of the presentation of this disease in the pediatric population. Laboratory tests, radiology studies, and endoscopic procedures are helpful in diagnosing inflammatory bowel disease and differentiating between Crohn's disease and ulcerative colitis. Once diagnosed, the goal of medical management is to induce remission of disease while minimizing the side effects of the medication. Specific attention needs to be paid to achieving normal growth in this susceptible population. Surgical management is usually indicated for failure of medical management, complication, or malignancy. Algorithms for diagnostic evaluation and treatment of pediatric inflammatory bowel disease are presented. The specific psychosocial issues facing these patients are also discussed in this review as are the future goals of research in the complex problem of pediatric inflammatory bowel disease.

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Key words: Pediatric; Inflammatory bowel; Ulcerative colitis; Crohn's disease

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INCIDENCE AND ETIOLOGY

The incidence of inflammatory bowel disease (IBD), which is most commonly divided into ulcerative colitis (UC) and Crohn's disease (CD), appears to be more common in countries in the northern hemisphere and in industrialized nations. The true worldwide incidence of pediatric IBD is unknown[1]. The annual incidence rates of 0.2-8.5 per 100 000 for CD and 0.5-4.3 per 100 000 for UC have been reported[2,3]. Some reports indicate that annual incidence of CD is continuing to rise and is approaching or surpassing that of UC[2,4,5]. The overall incidence of IBD increased significantly between 1960 and 1990[2,3]. In patients with IBD, 25%-30% of patients with CD and 20% of patients with UC are younger than 20 years old[4]. Although the incidence in children is lower than that in adults, it is also increasing with most recent estimates reported as 47 per 100 000.

The etiology of IBD is unclear. There are several theories but most agree that IBD is multifactorial. It is believed that a complex interaction of environmental, genetic, and immune factors lead to the development of IBD. The role of each of these components is believed to be different in UC and CD[6]. A combined immunogenetic model that encompasses all three is most likely[7]. Environmental factors including breastfeeding, smoking, diet, occupation, education, climate, and stress have been implicated. The most established association is smoking. Smokers are at a decreased risk for developing UC but at an increased risk for developing CD. The fact that it may be protective in UC has led to investigations of treatment regimens that include transdermal nicotine patches without conclusive results[5,6].

There is a genetic association, but not a clearly defined inheritance pattern. Frequency of IBD in first-degree relatives may be as high as 40%[6]. There is a higher rate of concordance for monozygotic twins with CD > UC compared with dizygotic twins. In UC, there is an increased frequency of HLA-A11 and HLA-A7 and a decreased frequency of HLA-A9 in CD. In Japanese patients with UC there is an increased frequency of HLA-DR2, but this is not seen in other populations[6]. Mutations in the NOD2/CARD15 gene on chromosome 16 have been associated with Crohn's Disease but not UC[7]. This gene is only expressed in monocytes and plays a role in activating nuclear factor-kappa B (NF-κB) which is a critical mediator of inflammatory responses and leads to the production of proinflammatory cytokines[8]. Patients with NOD2/CARD15 genotype have demonstrated fibrostenosing disease, usually in the ileum, and earlier disease onset[9]. It may be negatively associated with UC-like disease or the perforating type of CD[8]. Another gene,

Table 1 Frequency of presenting symptoms in IBD[2,12]

Symptom	CD (%)	UC (%)
Abdominal pain	62-95	33-76
Diarrhea	52-78	67-93
Weight loss	43-92	22-55
Hematochezia	14-60	52-97
Delayed growth	30-33	6
Fever	11-48	4-34
Perianal disease	25	0
Extraintestinal manifestations	15-25	2-16

IBD5, located within the 5q31 cytokine cluster, has been associated with perianal disease in Crohn's patients.

The immune system is also involved, but the inciting event is unclear. An infectious etiology has been considered and essentially all patients with IBD have increased titers against bacteria, viruses, and fungi. Of the many infectious agents that have been considered, including the virus that causes measles, *E.coli*, and M. paratuberculosis, no association of these has been proven[5,6]. Theories that focus on the involvement of the immune system include persistent bacterial infection, defective mucosal barrier, or an imbalance in the regulation of the immune response[7]. One theory of CD is described as an inappropriate immune response triggered by genetic or environmental factors[8]. There is some evidence that interleukin-15 (IL-15) and the IL-15 receptor (IL-15R) system is involved in inflammatory bowel disease. IL-15 is a cytokine with wide cellular distribution in monocytes, epithelium, endothelium, fibroblasts, and muscle cells. It plays a role in T cell proliferation and activation, the production of inflammatory cytokines in natural killer (NK) cells, and the migration of both. It also plays a role in the proliferation and differentiation of B cells. It is implicated in the pathogenesis and maintenance of several chronic inflammatory diseases with immune activation[10].

CLINICAL PRESENTATION

As with adults, the clinical presentation of IBD depends on the site and extent of mucosal inflammation[5,11]. At the time of presentation, 44%-49% of children with UC have rectosigmoid disease, 36%-41% have left-sided disease, and 14%-37% have pancolitis[2]. In terms of severity of illness, 50%-60% present with mild disease, 30% with moderate disease, and 19% with severe disease[4]. In children with CD, 50%-70% of pediatric patients present with disease involving the terminal ileum with more than half also having involvement of the colon, usually the ascending portion. Ten to 20% of children with CD have isolated colonic disease and 10%-15% have diffuse small bowel disease[2]. Gastroduodenal inflammation may be found in 30%-40% of patients with CD, but isolated gastroduodenal disease is rare, less than 5%[4]. The most common presenting symptoms in UC include weight loss, rectal bleeding, diarrhea, and abdominal pain. (Table 1) CD may have an insidious onset with abdominal pain and weight loss which may contribute to a delay in diagnosis.

Table 2 Manifestations of IBD[2,4,5,12,13]

System	Manifestation
Generalized	Fever
	Weight loss
	Malaise
	Anorexia
	Fatigue
	Nausea/vomiting
Ocular	Uveitis
	Episcleritis
	Iritis
	Conjunctivitis
Oral	Cheilitis
	Stomatitis
	Apthae
Pulmonary	Pulmonary vasculitis
	Fibrosing alveolitis
Vascular	Vasculitis
	Thrombosis
Hepatobiliary	Primary Sclerosing Cholangitis
	Hepatitis
	Cholelithiasis
	Jaundice
Pancreatic	Pancreatitis
Gastrointestinal	Abdominal pain
	Nausea/vomiting
	Diarrhea
	Hematochezia
Renal/Urinary	Nephrolithiasis
	Obstructive hydronephrosis
	Enterovesical fistula
	UTI
	Amyloidosis
Hematologic	Iron deficiency anemia
	Anemia of chronic disease
	Thrombocytosis
	Vit B_{12} deficiency
	Autoimmune hemolytic anemia
Endocrine	Decreased growth velocity
	Delayed sexual maturation
Integumentary	Erythema nodosum
	Pyoderma gangrenosum
	Perianal disease
Musculoskeletal	Osteopenia and osteoporosis
	Arthritis/Arthralgias
	Ankylosing spondylitis

If chronic diarrhea or rectal bleeding are present, diagnosis may be more expeditious. Non-specific symptoms of anorexia, fatigue, delayed sexual maturation, and decreased linear growth may be present but not immediately noticed and if noticed may not prompt a workup for IBD because of their nonspecific nature.

Extraintestinal manifestations are common in both adults and children with IBD, affecting 25%-35% of patients (Table 2)[4]. However, in children, these may precede the onset of gastrointestinal symptoms by years, whereas in adults these symptoms are usually concurrent with exacerbation of disease[4,5]. Arthritis is the most common extraintestinal manifestation occurring in 7%-25% of children with IBD[2,4]. Nephrolithiasis occurs in 5% of children with IBD, erythema nodosum occurs in 3%, and pyoderma granulosum in < 1%. Other extraintestinal manifestations include ocular complications, hepatobiliary complications such as primary sclerosing

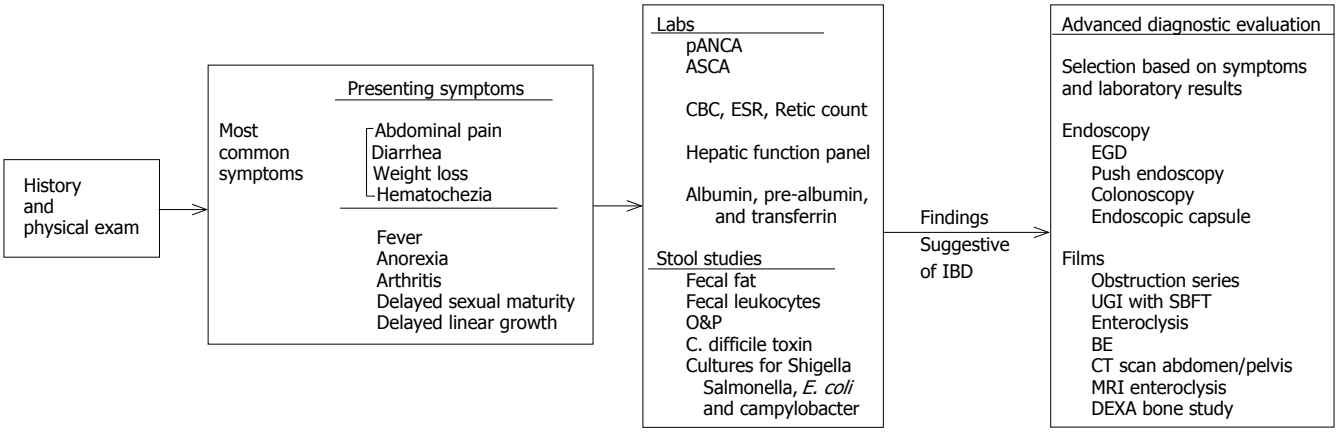


Figure 1 Diagnostic Evaluation for IBD in Pediatric Patients.

cholangitis and choledocholithiasis, and pancreatitis[4]. The differential diagnosis for IBD includes common causes of abdominal pain which includes constipation, gastroesophageal reflux, and peptic ulcer disease. In a population of children who are increasingly overweight, cholelithiasis must also be considered. Gynecologic causes of abdominal pain, such as ovarian cysts and torsion, must be considered in females. Infectious causes of abdominal pain including urinary tract infection, mesenteric adenitis, gastroenteritis, and appendicitis should also be included in the differential. Rectal bleeding can be seen with intussusception, infectious colitis, Meckel's diverticulum, colonic polyposis syndromes, hemorrhoids, and anal fissures. Diarrhea may be seen with gastroenteritis or infectious colitis. It is important to remember, however, that when these symptoms occur together or in the setting of weight loss, anorexia, growth failure, or other extraintestinal manifestations, the diagnosis of IBD must be considered and ruled out.

DIAGNOSTIC WORK-UP

History and physical exam are the initial steps in evaluating the patient with complaints suggestive of IBD (Figure 1). Important historical information includes recent infections, antibiotics, and travel history. Family history with specific attention to IBD diagnosis or related symptoms must be obtained. Without a thorough growth history to determine height velocity, growth failure might not otherwise be detected. Physical exam would include height and weight and calculation of the percentiles for each. Weight loss is present in 85% of children with CD and 65% of children with UC[2,4]. The abdominal exam may be normal or may demonstrate nonspecific tenderness. A mass in the right lower quadrant may be indicative of CD. A rectal exam is important to check for fecal blood, to evaluate for fissures or hemorrhoids, and to evaluate the surrounding area for fistulas or other perianal disease frequently associated with CD.

Laboratory evaluation is important to assess several aspects of patient status. First, serum markers for IBD should be checked[14]. Anti-*Saccharomyces cerevisiae* antibody (ASCA) and perinuclear antineutrophil cytoplasmic antibody (pANCA) assays are highly disease-specific for CD and UC, respectively, and can assist clinicians in diagnosing and categorizing patients with IBD[[11]. ASCA is associated with CD and if IgG ASCA and IgA ASCA are both detected, this is 100% specific for CD[8,11]. Higher ASCA levels are independently associated with onset at an earlier age and with fibrostenosing disease[8]. Positive titers for pANCAs are associated with UC in 60%-80% of cases[11]. Elevated titers can also be seen in left-sided or pan-colitis in children with CD[8,11]. When both tests are considered together, ASCA and pANCA have a specificity of 95% and positive predictive value of 96% for IBD[11]. A complete blood count (CBC), retic count, and sed rate should be checked to look for anemia and signs of acute or chronic inflammation. Albumin, pre-albumin, and transferrin should be checked as an assessment of nutritional status. Finally, stool studies should be performed to evaluate for evidence of malabsorption or infectious etiology. Cultures should evaluate for *C. difficile*, Salmonella, Shigella, *E.coli* (O157:H7), and *Campylobacter*.

Diagnostic imaging can provide valuable information in a patient whose history or physical exam is concerning for IBD. Plain films are important in the acutely ill patient to look for complications such as obstruction, toxic megacolon, or perforation. In the non-acutely ill patient, contrast studies may be more beneficial in making a diagnosis. A barium enema (BE) can identify strictures and abnormal mucosa. It is useful to evaluate the colon and often the terminal ileum if the contrast refluxes back through the ileocecal valve. An upper gastrointestinal (UGI) study with a small bowel follow through (SBFT) can evaluate the proximal gastrointestinal tract including small bowel which in the past has been difficult to assess. This study may show mucosal lesions, strictures, or fistulas. If a bowel obstruction is present, the transition point may be identified. Standard enteroclysis is considered superior to SBFT but is more difficult to perform and may not be tolerated by some patients. A computed tomography (CT) scan is helpful in the diagnosis and management of patients presenting with acute onset of symptoms or an exacerbation of their disease. CT can assess both intestinal abnormality and extraluminal abnormalities such as abscess formation. Ulcerative colitis is associated with mural

Table 3 Medications for IBD[4,12,19]

Class	Generic (Trade)	Use	Side Effects
Aminosalicylates	Mesalamine (Asacol, Pentasa) Sulfasalazine (Azulfadine) Olsalazine (Dipentum) Balsalazide (Colazal)	May be used to induce remission in mild CD or UC May be used to maintain remission in moderate CD or UC	Headache Nausea Anorexia Leukopenia Diarrhea
Corticosteroids	Prednisone Budesonide	Used for induction of remission in CD and UC, not effective in maintenance of remission	Increased risk of infection Hypertension Weight gain Acne Hirsuitism
Immunomodulators	Azathioprine (Imuran, Azasan) 6-mercaptopurine (Purinethol)	Used for maintenance of remission in moderate to severe CD or UC	Nausea/vomiting Diarrhea Pancreatitis Hepatitis Myelosuppression
	Methotrexate (Rheumatrex, Trexall)	May be used to maintain remission in moderate to severe CD or UC that does not respond to azathioprine or 6-mercaptopurine	Myelosuppression Oral ulcers Hepatitis
	Cyclosporine (Neoral, Sandimmune)	Has been used to induce and maintain remission in refractory cases of UC	Renal toxicity Hypertension Gingival hyperplasia Hirsuitism
Biologic Agents	Infliximab (Remicade)	May be used for refractory CD or steroid-dependent patients to induce and maintain remission	Infusion reaction (chest pain, Hyper- or hypotension, And shortness of breath) Nausea Fever/Chills Hives Fatigue

thickening < 1.5 cm, no thickening of the small bowel, increased perirectal and presacral fat, target appearance of the rectum, and lymphadenopathy. Findings consistent with CD include mural thickening > 2 cm, involvement of the small bowel, mesenteric fat-stranding, perianal disease, abscesses, fistulas, and adenopathy[15]. MR enteroclysis is a new technique being evaluated as a method to evaluate the small bowel for CD, and initial reports are promising that it is very sensitive. Thickening of the bowel wall, skipped lesions, stenotic areas, and fistulas have all been diagnosed with more sensitivity when compared to conventional SBFT. It has the added benefit of minimal radiation exposure due to the need for fluoroscopy to place a nasoduodenal tube prior to the study[16].

Finally, bone density studies should be considered either to establish a baseline or, if corticosteroid treatment is prolonged, to evaluate bone mineralization which can be altered in these children. It is important to interpret this study using bone age or height age as opposed to chronological age because using chronological age will lead to an overestimation of the extent of bone disease. Endoscopic evaluation is an essential tool in the workup of IBD. UGI endoscopy looks at the esophagus, stomach, and duodenum. It is useful in identifying other possible sources of fecal blood such as gastritis, duodenitis, or peptic ulcer disease. Using a push endoscopy technique, it may be possible extend visualization to 60-120 cm beyond the ligament of Treitz to evaluate for small bowel CD[17]. Biopsies can be taken to establish or confirm the diagnosis. Colonoscopy is a standard part of the workup for IBD. The advantage of this procedure over contrast studies is the ability to obtain biopsies which can distinguish between UC and CD involving the colon.

Biopsies taken during endoscopic evaluation help distinguish between CD and UC. There are several pathologic features which help distinguish between the two types of inflammatory bowel disease when the colon is the primary site of involvement. UC always involves the rectum and can extend proximally, whereas CD may involve any portion of the colon including isolated right colon involvement or multiple segments with skip areas in between. The microscopic features of UC include diffuse inflammation confined to the mucosa or submucosa. The microscopic features of CD include transmural inflammation with fissuring ulcers, fistulas, granulomas, and vasculitis[18]. The endoscopic capsule has been shown in several studies to have a higher yield than either push endoscopy or enteroclysis when used to evaluate patients with small bowel CD. One such study evaluated two groups of patients, those with the previous diagnosis of CD and those with no prior diagnosis. In both groups, the endoscopy capsule was more sensitive for small bowel pathology than either of the other two techniques[17]. Problems with this technique include difficulty in swallowing the capsule and failure to pass the capsule.

MEDICAL MANAGEMENT

The main goal of medical management for IBD is to achieve clinical and histiologic remission by suppression of inflammation with the least amount side effects from the medications. (Tables 3, 4) Good control of

the inflammatory process will decrease the likelihood of needing surgical intervention. In addition, successful treatment should provide adequate nutritional support to promote normal growth and should attempt to minimize alteration in the physical and social functioning of the child in order to maintain quality of life[1]. For patients with CD, treatment is based on severity of symptoms, which does not necessarily correlate with mucosal healing. CD is classified as mild to moderate, moderate to severe, and severe to fulminant. Mild to moderate disease is treated initially with either 5-aminosalicylic agents (sulfasalazine or mesalamine) or antibiotic therapy with metronidazole and ciprofloxacin[8,12,19]. Both regimens have been reasonably successful in inducing remission in mild disease in adults and children.

For patients with moderate to severe disease, corticosteroids are effective in inducing remission and have been considered the standard for treatment[4,8,12,19]. Prednisone or methylprednisolone remain the first-line therapy in moderately to severely ill children with CD with up to a 90% rate of improvement. The dose is 1-2 mg/kg per day prednisone equivalent (max 4060) for four to six weeks and then taper[4,8,19]. Although corticosteroids act rapidly to alleviate symptoms, they do not heal mucosal lesions and are of no benefit for maintenance therapy[5]. Pitfalls to this treatment include corticosteroid-dependence (1/3-1/2 of patients) and resistance (1/5 of patients) and therefore, weaning as quickly as possible is important to avoid decreased linear growth and minimize potential bone loss[8,19]. Steroids should be avoided in children with profound growth retardation, especially in those who are already Tanner 2 or 3 and who have a limited window of growth remaining[4,8,19]. They should also be avoided in those patients with severe bone loss.

In patients who have achieved remission with corticosteroids, immunomodulators, including azathioprine (AZA) and 6-mercaptopurine (6-MP), a metabolite of AZA, are helpful in maintaining remission[4,8,12,19]. Most pediatric gastroenterologists favor immunomodulators in children with CD as they are better at maintaining remission[8]. They may be initiated at diagnosis, post-op, after a severe attack. They are also used to maintain remission when patients have become steroid-dependent, but they are not effective in inducing remission because of their long onset of action. They have been shown to reduce steroid dependence and resistance from 50% to 20% or less[8]. Adverse effects include hepatotoxicity, myelosuppression, pancreatitis, N/V, and allergic reactions, and these have been associated with an inherited inability to metabolize them. Patients heterozygous or homozygous for an alteration in the gene thiopurine methyltransferase (TPMT) have deficient TPMT activity and are at risk for marked elevations of the active 6-thioguanine nucleotide metabolites and resultant severe leucopenia. Those patients with high TPMT activity can metabolize the drugs to inactive, but potentially hepatotoxic, 6-methylmercaptopurine metabolites. A screening test is available to determine TPMT genotype and activity as well as thiopurine metabolism[8].

Methotrexate (MTX) is another immunomodulator used as an alternative therapy after treatment with the thiopurines (AZA and 6-MP) has proven suboptimal due to inability to maintain remission or patient intolerance to the medication[8]. Methotrexate is also used in patients who do not respond to corticosteroids for induction of remission as it has been shown to induce and maintain remission[4,8,12,19]. Side effects include nausea, vomiting, diarrhea, stomatitis, rash, arthralgias, leucopenia, and hepatotoxicity. The latter is the most treatment- limiting and requires monitoring of serum aminotransferases and biopsy in patients with persistent elevations. There are also concerns of teratogenicity in females of child-bearing age[8].

Table 4 Medical management of IBD[4,12,19]

	CD	UC
Mild	Aminosalicylates Antibiotics	Aminosalicylates
Moderate	Corticosteroids→ Aminosalicylates or AZA or 6-MP or Methotrexate	Corticosteroids→ Aminosalicylates or AZA or 6-MP or Methotrexate
Severe	Corticosteroids→ AZA or 6-MP or Methotrexate or Infliximab	Corticosteroids→ AZA or 6-MP or Methotrexate or Cyclosporine
All cases	Nutritional support Monitor growth closely	Nutritional support Monitor growth closely

Finally, biologic therapy in the form of infliximab, a chimeric monoclonal antibody directed against tumor necrosis factor-α, has been used to treat moderate to severe disease in patients who do not respond to 5-ASA, antibiotics, steroids, or immunomodulators. It can be used as an alternative to prednisone to induce remission or as a first-line therapy in CD with severe, perianal, fistulizing disease or intolerance to corticosteroids[8,19]. Most of the literature is on adults but has been used in children with good results. Approximately 30% of patients do not respond to infliximab[5]. It is possible to develop antibodies to infliximab and thus decrease responsiveness. Concomitant therapy with immunomodulators may help maintain responsiveness[8]. It is generally well-tolerated by adults and children[4,8,19]. Side effects include flushing, rash, dyspnea, and headache and are typically seen at the time of infusion. Other side effects include delayed hypersensitivity reactions, including joint pain/stiffness, muscle pain, fever, general malaise, a lupus-like syndrome, and opportunistic infections, including tuberculosis, histoplasmosis, and sepsis. Leukopenia has been seen as well as lymphoproliferative disorders[5].

Patients with UC may be classified as having mild, moderate, or severe disease. In UC, mucosal healing correlates well with clinical remission[4,19]. The accepted treatment of mild to moderate UC is the 5-aminosalicylic acid agents, sulfasalazine and mesalamine. It is important to treat with appropriate dosing as underdosing is the most common cause of treatment failure. In UC, it is also important to remember that topical treatment with enemas is very effective, often more effective than oral therapy[19]. In patients with moderate to severe disease who are refractory to the aminosalicylates, corticosteroid treatment can induce remission but has no role in

maintaining remission[4,12,18]. The above side effects are still a concern, and the child should be tapered from corticosteroid therapy as soon as possible[4,19]. In patients with severe UC who fail to respond to corticosteroids, cyclosporine is an option for therapy[12,19]. This drug has a serious side effect profile, including renal insufficiency, hypertension, opportunistic infections, seizures, and electrolyte abnormalities[4,19]. Because of the toxicity of cyclosporine, the patient's history should be considered to assess whether surgery is a better option.

The immunomodulators AZA, 6-MP, and MTX have been studied in refractory cases of UC. They are not used to induce remission, but to maintain it[4,12,19]. Their side effects have been discussed above, and monitoring is required. As with the aminosalicylates, underdosing is frequently the cause of failure of therapy.

Aggressive nutritional support can improve growth parameters in pediatric patients with IBD[8]. Special diets have been shown to help alleviate symptoms in CD, but not in UC. However, all children with IBD need appropriate nutritional assessment and support to minimize growth retardation due to malnutrition. During acute exacerbations of IBD, parenteral nutrition may be necessary but has the associated disadvantages of being more invasive and more expensive[3]. In addition, patients receiving parenteral nutrition are at risk of complications from central venous access including infection and bleeding. Enteral nutrition is always preferable if tolerated. Formulas with omega-3 fatty acids may be beneficial[3]. Special attention should be paid to caloric intake and vitamin and mineral supplementation. During active disease, increased metabolic demands may make it difficult to compensate for the added caloric requirements with oral intake alone. IBD patients are also at risk of vitamin and mineral deficiencies, including iron, calcium, vitamin D, vitamin B12, and folic acid. Calcium supplements seem to be the best way to prevent bone disease.

SURGICAL MANAGEMENT

Surgical management of CD is typically required for complications of the disease. Indications include perforation/abscess, obstruction, stricture, fistula, toxic megacolon, or malignancy. Ultimately, greater than 50% of patients will require resection[20-22]. Surgical procedures are based on the location of the disease and the complication. The surgeon must consider the state of health of the patient. Obstruction is the most common cause for surgery in patients with CD. Adhesions are divided carefully, and strictures are either resected or managed with a strictureplasty[21-23]. Given the high rate of recurrence and reoperation, bowel conservation is extremely important to avoid the complications associated with short bowel syndrome and long-term dependence on parenteral nutrition. Strictures less than 10 cm in length can be addressed with a Heineke-Mikulicz strictureplasty. This is performed by incising the bowel lengthwise through the strictured area and closing the defect transversely. Longer strictures, 10-25 cm, may still be managed with strictureplasty using a Finney technique. Again, the bowel is opened in the longitudinal axis through the strictured area and a side-to-side anastamosis is performed. Strictures longer than 25 cm usually require resection. In pediatric patients with severe symptoms despite medical therapy or those with severe growth retardation, surgical resection of the terminal ileum and cecum may be beneficial in providing a disease-free interval of 2-3 years. Primary anastomosis is usually safe in the above mentioned circumstances. However, more severe complications, including perforation, abscess, or toxic megacolon, may require a resection and ostomy formation. Complications after surgery for CD include wound infection, anastomotic leak, anastomotic stricture, fistula, recurrence of disease, small bowel obstruction, and bleeding.

Crohn's disease is characterized by a chronic relapsing course. Recurrence of symptomatic disease after surgery has been reported as 17% at 1 year, 38% at 3 years, and 60% at 5 years. The disease typically recurs at the site of resection[20]. If defined as the presence of endoscopic abnormality, 1 year recurrence has been reported as high as 75%-93% of patients[20]. Growth improved significantly in pediatric patients after surgery[21,24]. Although there is a high rate of recurrence, any disease-free interval with decreased corticosteroid requirements may allow normal growth and sexual development. Risks and benefits must be clearly defined when determining the need for surgery[24]. There is some evidence to indicate that post-op management of CD patients with 6-MP can decrease rate of recurrence (recurrence defined as endoscopically-evident), but there is no standard of therapy[20]. Infliximab has not been investigated in this setting.

Ulcerative colitis may require surgery for emergent or elective indications. The former includes bleeding, toxic megacolon, and perforation. Indications for non-emergent surgery include failure of medical therapy, severe growth retardation, and dysplasia or malignancy. The rate of colectomy is no higher in children than in adults, but children have a higher rate of progression of disease (65% for children *vs* 39% for adults)[4]. Unlike in CD, total proctocolectomy for UC is curative. The procedure used depends on the clinical status of the patient but usually involves a total colectomy with or without a protective ileostomy. Creation of a reservoir in the small bowel with a ileoanal anastomosis is the definitive procedure. Outcome parameters of importance include number of bowel movements per day, consistency of the stool, requirement of stool-regulating medicine, whether patients can discriminate between air and stool, emptying of J-pouch, ability to defer defecation, leakage of stool/soiling, and number of episodes of pouchitis[25]. Complications include wound infection, pouchitis, SBO, pelvic sepsis, bleeding, urinary complications, anastoamotic leak or stricture, soiling, mortality, and permanent stoma, which is reported as high as 30%[25].

Whether to perform proctocolectomies as a one-stage or two-stage operation remains controversial. Many studies show that a total colectomy with J-pouch ileoanal anastomosis can be performed safely as a single-stage operation with the right patient selection and careful attention to signs of complications[21,26,27]. The largest patient series is one of 150 patients with a IPAA without a protective ileostomy compared to 92 patients who

had IPAA with a protective ileostomy[26]. Results were compared, and a significantly lower complication rate was found in the one-stage procedure. However, the exclusion criteria for the one-stage procedure placed the patients with the highest risk of complication in the two-stage procedure group[26].

TYPICAL COURSE OF DISEASE

Crohn's disease is unpredictable and is characterized by recurrent exacerbation of symptoms. The spectrum of disease includes patients that remain in remission, those that have recurrent periods of disease followed by remission, and those that have continuous active disease[5,8]. Serum markers, including ASCA and pANCA, may help stratify patients into subgroups, which help predict natural history[9,28]. ASCA IgG and IgA is found in 100% of CD patients with fibrostenosing disease, whereas pANCA was present in 86% of CD patients with primary colonic involvement. At the time of diagnosis with CD, 40.9% of patients had ileocolonic disease, 28.6% had disease limited to the small bowel and 30.4% had disease involving only the colon and perirectal area[5]. Patients with small bowel disease are more likely to have an obstructive pattern of disease characterized by fibrostenosis. Patients with colonic disease are more likely to have symptoms associated with bleeding and inflammation[5,8]. In general, surgery is for complications of the disease, and three out of four patients will ultimately require surgery. Complications of CD include abscesses, fistulas, strictures, and obstruction from adhesions, and pediatric patients have an even higher risk of developing these complications[5]. They are addressed surgically, with the goal of minimizing loss of length of bowel, since it is not a curative procedure. Removal of the diseased segments is followed by recurrence near the anastomosis. Endoscopic evidence of recurrence is found in up to 93% of patients at 1 year. However, a second operation is only required in 25%-38% of patients at 5 years and 50%-70% of patients at 15 years. Of those requiring a second operation, 37% will require a third one[5].

There is an increased risk of cancer in CD patients, including small bowel adenocarcinoma and colon cancer. The risk is especially high in those patients diagnosed prior to the age of 25 and in patients with a history of pan-colitis. CD patients with small bowel disease are 50-100 times more likely to develop small bowel adenocarcinoma[4]. At the time of diagnosis, most patients with UC have disease distal to the splenic flexure[2,5]. In general, clinical severity at the time of presentation correlates with extent of colonic involvement[2,5,11]. Most patients have a chronic, intermittent course of disease recurrence. Approximately 15%-40% of patients will ultimately require a colectomy, and this correlates with the extent of involvement. Indications for surgery include failure of medical therapy, growth retardation, and complications such as persistent hemorrhage, perforation, toxic megacolon, and the presence of dysplasia or carcinoma[4,5]. Toxic megacolon occurs in 5% of adult patients but is rare in children. It is a surgical emergency associated with risk of perforation, sepsis, electrolyte abnormalities, and hemorrhage. Frank perforation is more common in UC than in CD. It is usually associated with toxic megacolon and serious disease.

The association between UC and colon cancer is well documented[5]. Risk appears to be related to extent of colonic disease, age at diagnosis, and length of time since diagnosis with greater than 10 years being the threshold for identifying an increased risk of cancer[5]. The overall annual incidence of colon cancer in patients with UC of longer than 10 years duration is approximately 1%[5]. Children who develop UC before the age of 14 have a colon cancer incidence rate of 5% at 20 years and 40% at 35 years[4]. It is estimated that the risk of dying from colon cancer is 8% after 10 years from diagnosis[4]. The risk of colon cancer in patients with Crohn's colitis is 4-20 times that of the general population. Overall, patients with UC do not have a significantly increased mortality rate when compared to the general population. However, those patients with CD do have an increased mortality. Their survival rate is 93.7% of that in the general population at 15 years[5].

PROBLEMS SPECIFIC TO PEDIATRIC PATIENTS

Growth retardation was mentioned briefly earlier in this review but warrants further discussion. It may be present at the time of diagnosis or occur as a result of treatment[3]. Inadequate nutrition, chronic corticosteroid use, and decreased physical activity all play a role[3]. It is important obtain a complete growth history and follow growth parameters closely as growth failure may affect a patient' s treatment plan. Height velocity is the most important parameter to diagnose impaired growth, with more than 85% of patients having impaired growth at the time of diagnosis with IBD[4]. Nutritional insufficiency is due primarily to inadequate intake either from anorexia or food fear related to the association of food intake with pain[4]. Undernutrition delays skeletal and sexual maturation, with delays seen in epiphyseal fusion of long bones and in progression through Tanner stages[4]. Corticosteroids may further complicate growth and maturity. Factors contributing to abnormal bone mineralization over-production of inflammatory cytokines with the resultant increase in bone resorption, malnutrition, sedentary life-style, and corticosteroid use. Corticosteroids inhibit osteoblasts, impair intestinal absorption of calcium, and antagonize growth hormone[3]. Diagnosis of bone disease can be made with radiologic studies. A dual energy X-ray absorptiometry (DEXA) study is particularly useful. However, results should be interpreted using bone age or height age, not chronological age which overestimates the extent of bone disease.

In addition to the multitude of medical problems faced by pediatric IBD patients, there are significant psychosocial stressors for these children. Emotional and behavioral issues are common and stem from the chronic nature of the disease. Lifestyle changes can occur unexpectedly due to disease exacerbation. Anxiety, depression, and antisocial and dependent behavior are all common. Anxiety and depression may be related to concerns about

differences from other children and progression of their illness such as relapse of the disease or complications of disease requiring surgery or stoma formation[29]. Difficulty interacting with peers may result from school absenteeism and may be compounded by low self esteem due to delayed growth and development[2,30]. Finally, guilt may be a large psychosocial stressor for the child. The financial strain of a chronic illness, changes in parental work schedules, repeated physician visits, and sibling issues are problems for which a child with IBD may feel responsible. It is also important to remember the educational needs of children with IBD. Due to illness, attendance in school may suffer. If not anticipated and addressed, this can cause the child to fall behind in school. All of the above mentioned problems can be exacerbated if the educational needs of the child with a chronic illness are overlooked[3,29,30].

Assessment of a child's adaptation to his or her disease is a component of long-term care of children with IBD. Several assessment tools exist with most models focused on evaluating social support, coping skills, effects on activities of daily life, and body image[2,3,30,31]. These broad categories are used to assess quality of life and have been shown to be affected by age, education, and gender[30,31]. Finally, the issue of the transition of care is inevitable for children with IBD. After dealing with a chronic illness for, in some cases, years, medical care is transferred to a different provider. The child has built a relationship with the pediatric gastroenterologist and they must change physicians, perhaps at a time when many other changes are taking place. Preparing adolescents with IBD for this transition takes time and planning. This transition can be facilitated by the pediatric gastroenterologist by gradually increasing the patient's participation in, and responsibility for, his or her own care. Older adolescents should know their medications and be able to recognize signs of exacerbation of their disease. In addition, they should know the warning signs of an emergency and be able to take appropriate steps to seek immediate treatment. There are several recommendations for the pediatric gastroenterologist to help make this a smooth transition of care. These include: (1) preparing a detailed, up-to-date medical summary describing the course of the disease, therapy, allergies, *etc*., (2) including copies of relevant test results, and (3) identifying a skilled gastroenterologist who is aware of the special needs of the young adult patient who was diagnosed with IBD as a child[2]. The goal is for everyone involved to view this as a transition of care rather than a transfer of care. Maintaining open communication between the patient and the two physicians is critical for this to be successful.

Future considerations

The North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) has identified areas of interest for future research. These include the role of enteric microbial flora; the study of dietary, nutritional, and environmental factors; the evaluation of new therapies and their effects in early onset disease; and the development of a North American Pediatric IBD database[3]. The Working Group of the Second World Congress of Pediatric Gastroenterology, which includes NASPGHAN, has set goals of future research to include establishing the correlation between genotype and phenotype, the determining whether mucosal response in IBD differs between children and adults, understanding the role of previous exposure to infections, and improving the dissemination of knowledge regarding pediatric IBD[1].

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Surgical perspectives in gastrointestinal disease: A study of quality of life outcomes in esophageal, pancreatic, colon, and rectal cancers

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Abstract

Outcomes assessment in surgery traditionally has included data regarding peri-operative mortality and morbidity, as well as long-term survival and recurrence in surgical oncology. However, quality of life (QOL) is another important patient-related outcome measure. QOL data can be used to tailor treatment and improve clinical outcomes by detecting physical or psychological problems in patients that otherwise might be overlooked, but which have profound implications for the effective delivery of care. We review several well-validated QOL instruments developed specifically for use in patients with gastrointestinal malignancies, including esophageal, pancreatic and colorectal cancers, and conclude that QOL assessment routinely should be included in clinical trials of novel treatments.

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Key words: Gastrointestinal malignancy; Quality of life; Patient-related outcomes

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DESCRIPTION OF QUALITY OF LIFE

The assessment of quality of life (QOL) is a relatively new facet of health services research in surgery. Traditionally, surgeons measured success or failure based on perioperative morbidity and mortality. However, as technology has improved surgeons' ability to intervene safely in patients' lives, researchers have been compelled to determine whether improvement in survival translates into improvement in QOL. QOL is a nonspecific term that attempts to define the overall state of a person's life. There are subjective and objective components to the QOL specific to one person. For example, two people with the same health and economic status could perceive their respective qualities of life very differently. Discrepancies in QOL are influenced by the social and psychological status of the individual. Research tools have been developed to quantify both subjective and objective evaluations of quality of life (Q) in order to obtain a healthcare-related value of QOL. Measurement scales are based on social, somatic, psychological, and physical domains to obtain a value (Z). To be validated and used in trials, Z must be shown to equal Q (Figure 1)[1].

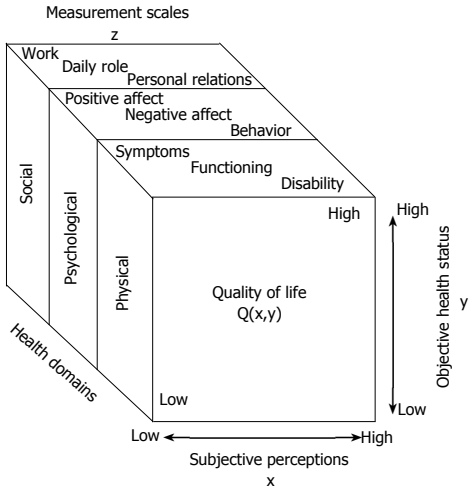


Figure 1 To measure QOL, social, psychological and physical domains of illness are assessed using measurement scales (z). QOL (Q) is dependent on both subjective and objective items. For a questionnaire to be validated, Z must equal Q. (Reprinted with permission from Testa *et al* N Engl J Med 334: 835-840, 1996).

QOL studies have increased rapidly in the medical literature, as demonstrated by the number of citations on Medline. In 2004, a keyword search on "quality of life" identified 5584 articles, whereas 20 years earlier (1984),

only 45 citations were elicited. QOL trials can discriminate between therapies with similar rates of morbidity and mortality, such as diverting ostomy or bowel resection with primary anastomosis in colon cancer, or compare different treatment modalities, such as medicine versus surgery. Assessment of QOL outcomes requires accurate gathering of information, use of pertinent measurement tools, and appropriate follow-up. A structured QOL questionnaire consists of a number of items in the form of questions or statements that draw on various dimensions of QOL . The form used to assess these subjective and objective data must be validated and then filled out either by the patient, a proxy, or by interview (telephone or face-to-face). The telephone interview limits follow-up to people who do not have hearing impairments and who have a telephone. Self-administered questionnaires give patients the opportunity to address problems they might otherwise be reluctant to discuss openly with an interviewer, but there is an increased risk of misinterpretation. Face-to-face interviews decrease misinterpretation but might fail to represent aspects of QOL people are embarrassed to discuss. Survey answers are assigned a score; qualitative information is converted into quantitative data, such that standardized scores are inclusive of all represented dimensions within the instrument[2].

Instruments calculate QOL in a generic or specific manner. Generic questionnaires provide an overall assessment of QOL that is not matched for a certain population or disease state. A popular, validated example of a generic QOL instrument is the Short Form-36 (SF-36). The questionnaire includes 36 items that measure eight dimensions of health on multi-item scales, including physical function, emotional function, physical problems, emotional problems, pain, general health, vitality, and social function. Sample questions include "Does your health now limit you in vigorous activities, moderate activities, lifting or carrying groceries?" and "Have you felt downhearted and blue?" There is also a single-item scale measuring changes in health during the previous year. The scoring scale ranges from 0 to 100, with 0 equal to death and 100 equal to perfect health. On the SF-36 scale, higher scores imply better health[3]. The SF-36 is useful for assessing health in a global manner; for example, the SF-36 has been used around the world to assess QOL in patients with a variety of different chronic diseases[4]. However, the SF-36 is often not sensitive enough to detect small differences in specific treatment groups with unique health care disabilities. Specific health-care instruments are tailored to detect these small differences in QOL when treatment groups are more homogeneous. These questionnaires are specific to a type of disease, population, domain, or symptom[5].

In 1980, the European Organization for Research and Treatment of Cancer (EORTC) started a research program to develop an integrated approach for QOL evaluation in patients with cancer participating in international clinical trials. The goal was to develop a core questionnaire incorporating a wide range of physical, emotional, and social health issues relevant to a broad range of cancer patients, irrespective of specific diagnoses, supplemented with diagnosis-specific (i.e. esophageal, pancreatic, and colorectal cancers) and/or treatment-specific modules. Currently, the EORTC QLQ-C30 is the most widely used disease-specific instrument for the assessment of QOL in cancer patients worldwide. The 30-item questionnaire incorporates five functional scales (i.e. physical, role/daily activities, cognitive, emotional, and social), three symptom scales (i.e. fatigue, pain, nausea and vomiting), and a global health and QOL scale. The single items assess commonly-reported symptoms of cancer patients (i.e. dyspnea, appetite loss, sleep disturbance, constipation, and diarrhea), as well as the perceived financial impact of the disease and treatment[6].

It is important to distinguish between the measurement of direct and indirect costs accrued by patients during the diagnosis, treatment, and surveillance phases of their diseases. Direct health care costs include expenses accrued during visits to physicians, as well as the costs of prescription medications, physical therapy, hospitalization, operative procedures, laboratory, and radiological tests. Indirect costs include the value of production lost to illness-related absence, such as the number of days absent from (paid and unpaid) work and days lost from housekeeping and other daily activities. Currently, large administrative databases compiled by insurance companies, hospitals, and other healthcare institutions are the most reliable source for cost estimation, but they do not include most indirect costs. Estimation of the financial impact of disease on patients is a difficult task. The QLQ-C30 addresses the financial impact of disease by asking "Has your physical condition or medical treatment caused you financial difficulties?" However, other studies have attempted to further quantify the financial impact in a more specific manner. Goossens *et al*[7]. studied the use of a patient cost diary to evaluate all indirect costs in 205 patients with fibromyalgia and low back pain over a two-year period. They found that when the diary was used in conjunction with periodic interviews, there was an accurate estimate of direct and indirect costs, as well as good patient compliance completing the diary. Additional studies have shown that the cost of disease treatment can be collected prospectively to assess for differences in treatment arms using a Collection of Indirect and Nonmedical Direct Costs (COIN) form[8]. In order to fully evaluate the impact of financial strain on QOL, these forms can be used in conjunction with the QLQ-30.

ESOPHAGEAL CANCER

Esophageal cancer is the second most common solid intrathoracic malignancy in the U.S., after lung cancer. The National Cancer Institute (NCI) estimates that 14 520 Americans are diagnosed annually with esophageal cancer, and 13 570 die of their disease.(www.cancer.gov) Management of esophageal cancer depends on the stage of disease at presentation and the general health of the patient; treatment options include surgery, chemotherapy, radiation therapy, multimodality therapy, or palliative care. The esophageal cancer model is a good example of how a generic questionnaire was developed and then refined to obtain a specific QOL instrument that targets patients with esophageal cancer and their disease-specific symptoms. One of the first studies to examine QOL in patients with esophageal cancer used the EORTC QLQ-30 instrument,

in combination with a linear dysphagia scale, to assess for disease-specific complaints. This study by Blazeby in 1995 followed 59 patients (33 post-esophagectomy and 26 post-palliative care) for a median of 16 wk. Overall, patients undergoing palliation had lower QOL scores. However, the palliation group was heterogeneous, in that some patients received esophageal stents and pain medicine, while others received only pain medicine. Symptoms of dysphagia captured on the linear dysphagia scale did not correlate well with global QOL scores acquired on the QLQ-30. The authors concluded that the QLQ-30 is a valid instrument for assessing outcomes after treatment only if an additional disease-specific measure was added to identify dysphagia, an important QOL outcome for patients with esophageal cancer[9].

As a result of this study, esophageal patients and medical experts were consulted to reform and create a new questionnaire. The EORTC QOL Group developed a disease-specific module (QLQ-OES24) for patients with esophageal cancer undergoing surgery, chemotherapy, radiotherapy, and/or endoscopic treatment[10]. Using the EORTC QLQC30 and the dysphagia scale from the EORTC QLQ-OES24, QOL was followed this time in a prospective longitudinal study of patients with esophageal cancer[11]. Blazeby *et al*[12]. followed patients who underwent esophagectomy (n = 55) or palliative treatment with chemotherapy ± stenting (n = 37). QOL scores were obtained before treatment and at regular intervals. Median scores were calculated for patients surviving more than two years after esophagectomy (n = 17), for patients surviving less than two years (n = 38), and for patients undergoing palliative treatment (n = 37). Six weeks after esophagectomy, patients reported worse functional, symptom, and global QOL scores than before treatment. In patients that survived at least two years from the time of treatment, QOL scores returned to pre-operative levels within nine months, but patients who died less than two years after surgery never regained their pre-operative QOL. In both surgery groups, dysphagia improved, and the improvement was maintained until death, or for the duration of the study. Patients undergoing palliative treatment had more advanced disease and were more physically compromised from the beginning of the study. Palliative intervention allowed these patients to maintain their compromised QOL until death. The study demonstrated that esophagectomy had a negative impact on QOL in the short-term. Patients surviving less than two years had an improvement in dysphagia, but not in overall QOL. The authors concluded that if the subset of patients that would not survive two years could be identified pre-operatively, esophagectomy should be avoided and palliation and/or stenting should be recommended instead. In the following years, the QLQ OES24 module was refined to more clearly elicit symptoms of dysphagia and other problems with eating, as they are especially important for patients with esophageal cancer. With further analysis, items with poor convergence were eliminated. Ultimately, the module was refined to four scales and six single items; the QLQ-OES18 is now the prevailing validated disease-specific QOL measure for esophageal cancer (Figure 2). It should be used in combination with the QLQ-C30.

The Rotterdam Symptom Checklist (RSCL), developed in 1990, collects information about physical symptoms after surgery specific to esophageal cancer, including "dysphagia, loss of taste, weight loss, early satiety, blown-up feeling, hoarseness, pain behind the chest bone, food not going down, and nocturnal coughing[13]. A recent study by de Boer *et al*[14]. used the validated RSCL in combination with the Medical Outcomes Study Short Form-20 to assess QOL. Patients were randomized to receive a transhiatal (THE) or transthoracic esophagectomy and were followed for three years. Three months after surgery, patients in the THE esophagectomy group (n = 96) reported fewer physical symptoms (P = 0.01) and better activity levels (P < 0.01) than patients in the transthoracic group (n = 103). However, this difference was not sustained, and at longer follow-up there was no significant difference between the two treatment groups.

Answer question from 1-4 (1: Not at all; 2: A little; 3: Quite a bit; 4: Very much)
Could you eat solid food?
Could you eat liquid or soft food?
Can you drink liquid?
Have you had trouble swallowing your saliva?
Have you choked when swallowing?
Have you had trouble enjoying your meals?
Have you felt full up too quickly?
Have you had trouble eating?
Have you had trouble eating in front of other people?
Have you had a dry mouth?
Have you had problems with sense of taste?
Have you had trouble with coughing?
Have you had trouble with talking?
Have you had acid indigestion or heartburn?
Have you had trouble with acid or bile coming into your mouth?
Have you had pain when you eat?
Have you had pain in your chest?
Have you had pain in your stomach?

Figure 2 The EORTC QLQ-18 is a specific module for esophageal cancer. Patients complete the questionnaire based on QOL symptoms experienced within the last week. (Reprinted with permission from the EORTC.)

Recent trials have shown that clinical outcomes from esphagectomy can be improved by administering neoadjuvant chemotherapy[15]. In 2005, Blazeby examined the effects of neoadjuvant chemo-and radiation therapy on QOL in patients with esophageal cancer. Data were collected using the EORTC QLQ-30 in tandem with the cancer-specific QLQ-OES18. Patients receiving neo-adjuvant chemotherapy (n = 48) or chemoradiotherapy (n = 34) were noted to have a decline in QOL during treatment that was restored by the time of surgery. Patients who received neoadjuvant chemo-radiation had a relative improvement in post-operative nausea, dysphagia, and emesis compared to patients who underwent esophagectomy alone (P < 0.01). The authors concluded that neoadjuvant therapy improves disease-specific survival and potentially improves postoperative QOL[16].

At Yale, we are conducting a prospective cohort study measuring QOL in patients with esophageal adenocarcinoma who undergo THE with or without neoadjuvant chemo-and/or radiation-therapy. The specific aim of the study is to better select patients who will benefit from this type of surgery by identifying demographic and clinical predictors of QOL following

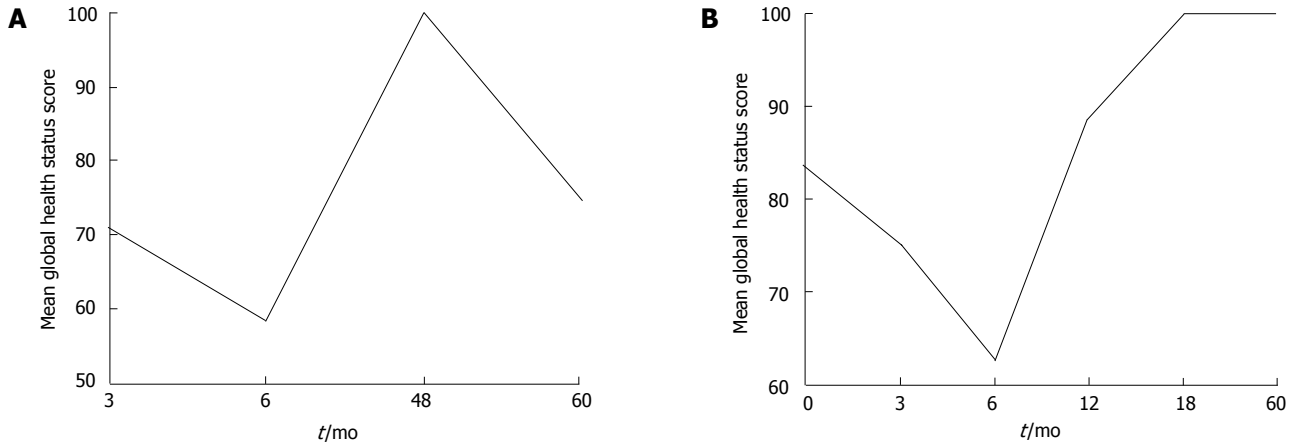


Figure 3 **A:** QOL after THE in patients with esophageal cancer ⩾ 70 years of age. QOL declines sharply for six months and then improves, but the recovery is not durable; **B:** QOL after THE in patients with esophageal cancer ⩽ 55 years of age. QOL declines for six months, but then improves in a more sustained fashion.

THE. The EORTC QLQ-OES18 is administered prior to surgery and then at regular intervals until death or for five years. Preliminary results for the first 21 patients demonstrate a dramatic decrease in global QOL scores in the first six months following surgery, with a gradual improvement in the following year (unpublished results). When comparing age groups following THE, the global QOL score of patients over age 70 years improves after the first six months, and then declines after two years (Figure 3A). In contrast, patients 55 years or younger improve their QOL to pre-operative levels by six months, and these improvements appear to be durable (Figure 3B) The trends in specific symptom scores (i.e. fatigue, nausea and vomiting, pain, and loss of appetite) are variable: they rise and fall during the first year, ultimately declining steadily by the fourth post-operative year. Dysphagia and reflux scores, with a range of 0 (no dysphagia or reflux) to 100 (complete dysphagia or reflux), plateau within the first year; the dysphagia score drops to 0, and the reflux score peaks (mean score, 45) at 18 mo. Functional scores (i.e. physical, emotional, and social functioning) nadir six months following surgery and then rebound to peak at four years; however, scores steadily decline by the fifth year after surgery.

More patients must be enrolled to better characterize the relative influences of such demographic and clinical factors as patient gender, race, tumor stage, comorbidities, and the use of neo-adjuvant and/or adjuvant treatment. Still, from our early data, we would conclude that candidates for THE should be counseled pre-operatively about the effects of surgery on QOL. The association between age and QOL outcomes should be considered when advising patients to undergo THE; that is, patients over age 70 should have a life expectancy of at least six to twelve months post-operatively to warrant the profound effects of THE on QOL.

COLON AND RECTAL CANCER

Colorectal cancer is the third most common cancer in the United States. The American Cancer Society estimates that in 2005, there will be 148 300 new cases of colon and rectal cancer, and 56 290 people will die of their disease (www.cancer.org). Generally, surgery is the initial therapy for patients with colon and rectal cancers. There are two prevailing validated QOL assessment tools for colon and rectal cancer: the Functional Assessment of Cancer Therapy-Colorectal (FACT-C) and the EORTC QLQCR 38, which like the QLQ-OES18 for esophageal cancer, is designed for use with the EORTC QLQ-C30. These instruments have been used in patients undergoing palliative or surgical treatment, regardless of the stage of their malignancy. The EORTC QLQ-CR 38 is a validated, reliable instrument that consists of 38 items inquiring about symptoms specific to colorectal disease and treatment, such as body image, sexuality, urinary problems, defecation or stoma, and future outlook on life[17]. The FACT-C was developed around the same time as the EORTC QLQ-CR 38. It is a shorter questionnaire, composed of 36 items from the core Functional Assessment of Cancer Therapy (FACT-G) merged with the colon and rectal specific items (CCS). These items were developed from interviews with colorectal patients and caregivers. They include two stoma-related questions. In 1999, Ward *et al*[18]. tested the FACT-C in English and Spanish-speaking patients with colorectal disease and found them to be reliable and valid. The authors concluded that the FACT-C had good internal consistency, reliability, and concurrent validity by addressing specific concerns related to colorectal cancer. In addition, in the Spanish population, it elicited subtle differences in QOL based upon extent of disease.

In a recent study by Rauch *et al*[20], 121 patients who received surgery for rectal cancer and who survived at least two years without evidence of disease recurrence were identified. These rectal cancer survivors were mailed three questionnaires: the EORTC QLQ-C30, the QLQ-CR38, and the Duke questionnaire. The Duke health profile is a 17-item validated instrument to assess QOL, but it is not specific for colorectal cancer[18]. The study patients reported less pain when compared to historical controls drawn from the general German and Norwegian populations ($P = 0.002$). Stoma patients reported better social functioning than did non-stoma patients ($P = 0.005$), with less anxiety ($P = 0.008$) and higher self-esteem ($P = 0.0002$). However, this was only detected on the Duke health profile, whereas the QLQ-30 did not demonstrate any difference. It is unclear whether the more generic

Duke profile captured some confounding effect on QOL (i.e. the positive effects of successful medical treatment), or if the EORTC module was too specific and failed to demonstrate real differences in QOL[19].

A recent study by Holloway *et al*[21]. from the University of Texas examined QOL as a predictor of post-operative length of stay (LOS) in 70 patients with colon and rectal cancers using the FACT-C module[20]. Patients completed the FACT-C questionnaire before treatment. LOS for patients scoring in the lowest quartile on the FACT-C in the pre-operative setting was compared with LOS for patients in the remaining quartiles. Patients in the lowest quartile of FACT-C scores had significantly longer LOS. Specifically, lower pre-operative FACT-C scores for physical well-being (9.1 *vs* 7.3 d; $P = 0.04$), functional well-being (9.6 *vs* 7.1 d; $P = 0.006$), and colon cancer concerns (9.5 *vs* 7.1 d; $P = 0.01$) correlated with increased LOS. Advanced patient age and surgical morbidity also predicted longer LOS, but additional factors such as race, nutritional status, stage of disease, and relative training of the operating surgeon did not appear to affect LOS. Courneya *et al*[22]. from Canada utilized the FACT-C on QOL in a prospective randomized-controlled trial to measure the effect of exercise on QOL after any type of colorectal surgery. Patients were assigned in a 2:1 ratio to either an exercise ($n = 69$) or control ($n = 33$) group. Exercise consisted of a home cardiovascular workout lasting 20-30 min, 3-5 times/wk; subjects had to reach at least 65% of their predicted target heart rate. Compliance was not good; therefore, analysis was limited to a comparison between patients who increased physical fitness to patients who had no change or decreased fitness. There was a significant improvement in QOL based on FACT-C scores between patients with increased physical fitness when compared to patients who did not increase physical fitness[21].

In 2005, Jung Yoo *et al*[23]. administered the FACT-C to 98 patients with colorectal cancer at the time of any surgery for rectal cancer. Fifty-two patients completed the survey at one and six months post-operatively; 38% received a stoma as part of the cancer operation. Analysis of variance between FACT-C scores demonstrated that patients' QOL was nadired one month after colectomy and returned to pre-operative levels six months later. This is an example of how QOL analysis can provide useful nformation for the patient regarding the projected post-operative course, which allows them to have some realistic expectations for recovery time and QOL[22].

PANCREATIC CANCER

Pancreatic cancer is the fourth leading cause of cancer-related death in males and the fifth leading cause in females in the Unites States (www.cancer.gov). It is usually diagnosed at an advanced stage, so survival is poor. There has been little change in overall incidence or mortality rates over the last three decades. Fewer than five percent of patients survive five years from the time of diagnosis. Whipple first described the modern pancreaticoduodenectomy (PD) in 1935. It remains the standard surgical treatment for adenocarcinoma of the head of the pancreas. Peri-operative mortality has diminished significantly, with experienced centers reporting rates of less than 5%. However, complications continue to be common and include pancreatic fistula, delayed gastric emptying, intra-abdominal abscess and hemorrhage, and anastomotic leak. Therefore, QOL studies in pancreatic cancer patients could play a significant role in determining appropriate treatment options. The EORTC QLQ-30 module was modified to identify symptoms that are unique to patients with pancreatic cancer. Over a two-year period, the EORTC study group on QOL performed literature searches, interviewed patients and health-care professionals, and identified 26 items related to pancreatic cancer that resulted in the QLQ-PAN-26 instrument[23]. A study by Shaw *et al*[25]. in 2005 examined long-term QOL following PD for benign and malignant disease[24]. The treatment group included 40 patients who underwent PD for benign and malignant disease. The benign group included patients with chronic pancreatitis, neuroendocrine tumors, pseudopapillary tumors and microcyst adenomas. The control group consisted of 58 patients who underwent cholecystectomy by the same surgeon. The two groups were matched for age and gender. Patients were followed for nine years. QOL was assessed using the EORTC QLQ-C30 and the QLQPAN26. While there was no significant difference in global health scores between the two groups, the QLQ-30 identified significant decreases in the post-operative functional scales (physical role, emotional role, and social) amongst the study PD group as compared with the control group. The malignant PD group reported the lowest level of physical and role functioning, greater weakness and concerns for future health, while the benign group had more social/financial difficulties, and upper gastrointestinal symptoms consistent with exocrine insufficiency. Upper gastrointestinal symptoms posed greater difficulties for patients who underwent total pancreatectomy including absolute dependence on pancreatic exocrine supplements and insulin-dependent diabetes. Patients after a PD had more diarrhea ($P < 0.05$) and nausea and vomiting ($P < 0.05$) than the matched patients after cholecystectomy. In part as a result of this study, patients are routinely placed on pancreatic enzyme supplements following PD at this research hospital.

A 2005 prospective longitudinal study by Nieveen van Dijkum *et al*[26]. assessed QOL in 114 patients following PD and a double-bypass procedure (hepaticojejunostomy and gastroenterostomy) for pancreatic and periampullary carcinoma[25]. The study employed the Medical Outcomes Study (MOS) 24 acute-phase questionnaire, the disease specific Gastrointestinal Quality of Life Index (GIQLI), and one question concerning overall QOL derived from the RSCL[13]. The GIQLI is an instrument developed in 1995 that has been validated in many patients with gastrointestinal diseases[26]. The questionnaires were administered over one year, or until death. A transient decline in physical (MOS score) and gastrointestinal (GIQLI score) functioning were observed at two weeks after surgery for both treatment groups. PD patients regained QOL within six weeks of surgery. Double bypass patients did not return to the pre-operative QOL until three months after surgery. There was a more pronounced

decline in all QOL scales after double-bypass procedures as compared with PD. The authors concluded that PD maintains QOL in patients with resectable pancreatic cancer. Palliative double bypass procedures have a beneficial effect on QOL in patients with unresectable pancreatic cancer that are expected to survive at least three months[27].

In conclusion, QOL studies seek to quantify symptoms and perceptions patients have in a generic- or disease-specific manner. Validating subjective and objective data has been labor-intensive; a large body of the work to date has been initiated by the EORTC. The EORTC QLQ-C30 has been used to measure QOL among patients with cancer in a reliable and reproducible fashion. However, it is important to include specific modifiers relevant for different disease processes. For example, dysphagia is measured in esophageal cancer, bowel habits in colorectal cancer, and bile duct obstruction in pancreatic cancer. Historically, post-operative morbidity and mortality have been the barometers used to assess clinical outcomes from different surgical interventions. Interestingly, economic outcomes, such as hospital charges and length of stay have also been reported. QOL is yet another outcome measure that should be collected when gauging the overall appropriateness of a surgical intervention. QOL information should be conveyed to patients in the pre-operative setting, so that they can make more informed decisions regarding their healthcare. We believe future research and clinical trials in surgery options in patients should routinely include analysis of QOL as an outcome measure.

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TOPIC HIGHLIGHT

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Evolution of surgical skills training

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Abstract

Surgical training is changing: one hundred years of tradition is being challenged by legal and ethical concerns for patient safety, work hours restrictions, the cost of operating room time, and complications. Surgical simulation and skills training offers an opportunity to teach and practice advanced skills outside of the operating room environment before attempting them on living patients.

Simulation training can be as straight forward as using real instruments and video equipment to manipulate simulated "tissue" in a box trainer. More advanced, virtual reality simulators are now available and ready for widespread use. Early systems have demonstrated their effectiveness and discriminative ability. Newer systems enable the development of comprehensive curricula and full procedural simulations.

The Accreditation Council of Graduate Medical Education's (ACGME) has mandated the development of novel methods of training and evaluation. Surgical organizations are calling for methods to ensure the maintenance of skills, advance surgical training, and to credential surgeons as technically competent.

Simulators in their current form have been demonstrated to improve the operating room performance of surgical residents. Development of standardized training curricula remains an urgent and important agenda, particularly for minimal invasive surgery. An innovative and progressive approach, borrowing experiences from the field of aviation, can provide the foundation for the next century of surgical training, ensuring the quality of the product. As the technology develops, the way we practice will continue to evolve, to the benefit of physicians and patients.

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INTRODUCTION

"See one, do one, teach one" is one way to paraphrase what has been the traditional teaching method of surgical education in the operating rooms of the United States for over a hundred years. This longstanding dogma is being increasingly challenged by legal and ethical concerns for patient safety, 80-h work week training restrictions, the cost of operating room time, and surgical complications. The emerging field of surgical simulation and training offers an opportunity to teach and practice laparoscopic skills outside of the operating room environment. There is enormous potential to address patient safety, risk management concerns, OR management, and work hours requirements with more efficient and effective training methods. The current goal of simulator training is to help trainees acquire the skills needed to perform complex minimally invasive surgical procedures prior to practicing them on living patients. For abdominal surgeons, minimally invasive surgery primarily involves laparoscopic techniques. The concept of minimally invasive surgery also applies to thoracic, gynecologic, head and neck surgery, orthopedics, or any other field where the size of the incisions and the degree of injury to the patient can be minimized with advanced techniques and equipment.

Novel methods of developing these skill sets are necessary because it is becoming increasingly clear that minimally invasive surgery requires a completely different skill set from the one used in traditional, open surgery[1-3]. Here spatial relationships and associated psychomotor skills are necessary to manipulate surgical instruments on a two-dimensional video screen in an actual three-dimensional operative field[4,5]. Developing ambidextrous skills in the small intraabdominal space, handicapped by instruments that have limited degree of freedom[6], while compensating for difficult camera angles and the fulcrum effect[7] often seems to be an overwhelming task for novices in the field of laparoscopic surgery. Unfortunately, even with the restrictions on work hours, we are now teaching residents at least two ways of performing each procedure, essentially doubling the skills that have to be learned over a five year residency.

Over the last few decades laparoscopic procedures have evolved from diagnostic laparoscopy[8] to advanced, more complex procedures. It is now routine for minimally invasive techniques to be used for bariatric, colonic, and advanced foregut procedures. These more advanced procedures require highly developed psychomotor skills. The surgeon's anatomic awareness must be developed in concert with the ability to safely achieve exposure and identify and control important structures. To assist with the critical components of many advanced operations, specialized equipment is available and commonly used. These instruments require a great deal of expertise in order to be used effectively and safely. Anecdotal reports have described significant complications related to the incorrect use of such equipment[9,10]. These developments have not gone unnoticed. The Food and Drug Administration (FDA) has now inserted itself into the training debate by requiring the development of a simulator-based training program (and that physicians demonstrate competence on the device) before approving a novel vascular stenting device. This ruling, in April of 2004, represents the first mandate by the FDA to require simulation training[11] without fully established or accepted data proving the validity of this approach. Similar requirements at the federal level for other advanced devices and skills sets are likely to follow. Payer-mandated and hospital-based credentialing for procedures and equipment is undoubtedly not far behind.

Surgeons who teach and train advanced procedures must now help develop methods of training and evaluation that truly establish procedural based competency. The tools are being developed and it is up to physician-educators to maximize their potential and set the standards for excellence. In a healthcare environment focused on evidence-based medicine and outcomes data, surgeons should set the standards rather than have them imposed by outside interests. The bar has already been set; the Accreditation Council for Graduate Medical Education (ACGME) now requires that program directors provide a statement attesting to the competency of every graduating Chief Surgical resident[12]. Many tools are now available to help with this task. The technology exists to continue to improve the realism and scope of training devices. Standardizing training routines and the effectively integrating systems for evaluation and credentialing is becoming a realistic goal. Below we will review currently available training and evaluation devices for laparoscopic surgery and discuss current future implications for training curricula for surgical residents.

BOX TRAINERS

This type of surgical simulator uses real surgical instruments and equipment including video monitors, cameras, and laparoscopes (Figure 1). It is an opaque box that approximates the size of the adult human abdominal cavity. Slits are prefabricated on the anterior surface of the box, through which trocars (access ports) may be placed. An attached flexible arm acts as a camera holder. Laparoscopic instruments are then inserted through the ports and into the box. Various targets are manipulated inside the box, with visual information relayed through a video source and display comparable to that used in most operating theatres. Tactile feedback is limited, as it is in laparoscopic surgery, by the instruments used. The use of real instruments and equipment is clearly the strength of these systems. However, the drills developed lack the face validity offered by other systems; the instruments may be real, but the "tissues" used clearly are not.



Figure 1 Typical Box trainer with real surgical instruments and equipment including a video monitor, camera, and laparoscope.

Many drills have been developed for use on these trainers. These drills traditionally involve movement and coordination exercises using peg boards or small objects such as beans or sugar cubes. Suturing skills are practiced using standard sutures and needles through plastic drains or other simulated tissue. Cadaveric tissue specimens can be placed inside the box to improve the realism of some tasks, particularly suturing. Practicing skills and tasks in the box trainer that resemble parts of real operations has been shown to stimulate the learning of psychomotor skills[13]. The physical sensory feedback conferred via the instruments in a box trainer is equivalent to that of surgery. The feel of the instruments on the surfaces of tissue, the pressure on closing a handle, and the compliance of compressed structures evokes feedback sensations called haptics[14]. This sensory feedback is one of the important attributes of the box trainer simulator.

Low acquisition cost is another key attribute of these devices. This makes the box trainer the most widely available training system. Its cost and availability have helped establish this type of simulator as one of the most validated systems in surgical training and evaluation. The use of these trainers has been well documented as part of the McGill Inanimate System for Training and Evaluation of Laparoscopic Skills (MISTELS)[15]. This system has been extensive studied and validated and has evolved into the Society of Gastrointestinal and Endoscopic Surgeons (SAGES) Fundamentals of Laparoscopic Surgery (FLS) course and evaluation system[1]. The FLS course combines an interactive CD-ROM educational tool that emphasizes basic principles of laparoscopic surgery with training drills for the box trainer.

The box trainer provides the backbone of the FLS evaluation tool. The FLS exam combines a written exam with a timed and scored laparoscopic skills evaluation in the box trainer. The tasks used for laparoscopic skill evaluation are peg transfer, pattern cutting, placement of a ligating loop, intracorporeal and extracorporeal knot

tying[15,16]. SAGES is currently recommending that all senior residents demonstrate laparoscopic skill competency by taking this exam. An examination fee is charged and, currently, the exam is only offered at six North American testing centers. The exam can also be taken at the yearly American College of Surgeons (ACS) Clinical Congress and the SAGES annual meeting.

The need to take a box trainer-based exam at a specialized test center demonstrates the major disadvantage of this system. The measurement of performance and objective evaluation of skills, or metrics, requires an independent teacher/evaluator[17]. For FLS, objective evaluation is performed with trained test proctors who time performance and apply penalties to the performance based on standardized criteria[18,19]. These criteria have not been published or made widely available, limiting the use of this validated evaluation system to only the approved test centers. As such, the FLS system is not useful as a routine evaluation tool for individual residency programs.

ANIMAL MODELS

These simulators involve the use of a live, anesthetized animal. This is the most realistic, non-patient environment for laparoscopic training. The abdomen in the porcine model is comparable in size to the adult human, with much of the foregut anatomy similar to that of the human[20]. Performing a cholecystectomy in this model provides tactile feedback in an environment where technical errors and complications such as gallbladder perforation or common bile duct injury can occur without consequence to a human patient[21]. Likewise, the canine model is frequently used to practice colon surgery. Animal models also enable trainees to work together as a team on an operation, providing additional insight into setting up an operative case.

As useful as animal labs are, there are many reasons why they are not fully integrated into most surgical curricula. Ethical issues regarding the use of animals for training and studies are not to be discounted, but for most programs the cost issues are prohibitive. There are substantial costs associated with maintaining specialized facilities and providing appropriate staff[22]. In some institutions, including our own, these facilities have been converted into an inanimate skills training lab.

VIRTUAL REALITY SIMULATORS

Virtual reality (VR) surgical simulators are the latest and most promising development in the area of surgical simulation. Sophisticated computer software has been developed in an attempt to replicate critical skills required for laparoscopic surgery and, in some cases, entire procedures. Many of these simulators provide a more believable practice environment than traditional box trainers, hence providing higher face validity. These trainers can be set up to record and save accurate and objective data for individual performance on specific tasks. The metrics of most devices can be customized, setting pass/fail criteria. These features present the opportunity for a trainee to practice independently on their own time as part of a structured curriculum.

The performance records make it possible for the educator to evaluate the performance of a laparoscopic task in an easy accessible format, to track the progress of an individual, and to compare a trainees results to peers and an expert standard[6,23,24]. Virtual reality simulators are also available for technically challenging tasks such as upper and lower endoscopy, endoscopic retrograde cholangiopancreatography (ERCP), hysteroscopy, and cystoscopy[24-28]. Objective measurements such as the time to complete a task, economy of hand motion, dexterity, and instrument path length can be easily used as assessment tools to document the progress of laparoscopic skills. The software of new VR simulators can be adjusted to create more difficult tasks or different levels of difficulty of the same task. Software updates have the potential to provide new drills or procedures which can be simulated using the same hardware. In an attempt to replicate the biggest advantage of box trainers and make the simulations as real as possible, several VR simulators now offer built in haptics, or force feedback, as an option on their systems. These systems, while intriguing, have not yet been shown to significantly contribute to training[29] but do significantly contribute to the cost of the devices.

Currently the following VR simulators are commercially available as training platforms for laparoscopic surgery: Procedicus MIST[30], Haptica ProMIS[31], METI SurgicalSIM, Simbionix Lap-Mentor and Surgical Science LapSim[32]. The two most widely used VR simulators are described in more detail below. The Procedicus MIST (Minimally Invasive Surgical Trainer), the most recent version of the MIST-VR (Mentice AB, Gothenburg, Sweden), uses a desktop computer with an interface similar to two laparoscopic instruments[7]. Twelve different tasks designed to improve hand-eye coordination and to stimulate psychomotor skills constitute the basis of this system. The complexity of these tasks ranges from basic grasping targets to transferring them between instruments to performing these tasks with an increased difficulty level, including the use of diathermy[33]. Recent updates to the system have incorporated suturing and knot tying drills. The MIST system remains the most extensively validated virtual reality training system. Seymour *et al*[34] demonstrated that surgical trainees who practiced a diathermy task to an expected level of performance performed faster and with fewer errors in excising the gallbladder off the liver.

The LapSim system (Surgical Science, Göteborg, Sweden) (Figure 2) also consists of laparoscopic instruments (VLI interface, Immersion Medical, Gaithersburg, MD) connected to a desktop computer (Figure 2). Similar to the MIST, the LapSim focuses on basic laparoscopic skills and suturing. Newer software modules incorporate complex diathermy and bowel manipulation. The LapSim system has different modules mimicking different tasks in the abdominal cavity focusing on camera navigation, instrument navigation, coordination, grasping, cutting, clip applying and more advanced modules like suturing and "running the small bowel." The simulator records data on multiple parameters for each module including "pass/fail," tissue damage and excess instrument motion[35,36]. All of the various data

sets are easily accessible and downloadable with the latest software release. This same software allows a teacher to set up specific drills and tasks as part of a curriculum for each trainee. All the metrics of this system are customizable. Likewise, performance exams can be tailored to skill level or individual requirements. Performance on the LapSim system has been demonstrated to distinguish expert and novice skill levels. This establishes construct validity for this system[36]. Specific skills transfer to the operating room has not yet been demonstrated.

FULL PROCEDURAL SIMULATORS

Full procedural simulators go beyond the basic laparoscopic training of their predecessors and are designed to recreate the specific anatomy allowing the practicing of all skills necessary to perform a particular operation. There are a variety of simulators available for endoscopic sinus surgery, bronchoscopy, endovascular surgery, gastrointestinal endoscopy, and laparoscopic procedures such as cholecystectomy and inguinal hernia repair[37-40]. Many of these simulators are part of a computer-based virtual reality platform. The hernia repair simulator is a physical model simulator[39].

The potential advantage of full procedure simulators over basic skills simulators is obvious. A well designed full procedural simulation should teach skills, anatomy, and the nuances of a complete surgical procedure in an environment where errors can be made without consequence. A full procedure simulator will not replace basic skills drills but will help bridge the gap to the operating room. For instance, in a laparoscopic total extraperitoneal hernia repair (TEP) even an experienced surgeon might encounter difficulties during repair due to the different approach and the unusual view of the inguinal anatomy. A full procedural TEP-simulator in the training of surgical residents resulted not only in improvement of operative performance but with their new understanding the residents were also more willing to recommend this procedure to patients requiring an inguinal hernia repair[39].

TRAINING OF SURGICAL RESIDENTS

In 1999 the Accreditation Council of Graduate Medical Education (ACGME) endorsed the concept of six core competences which every physician should demonstrate: Patient care, medical knowledge, practice-based learning and improvement, interpersonal and communication skills, professionalism, and systems-based practice[41]. These competencies are now the basis of individualized evaluations in most residency training programs. These competencies do not directly address the acquisition of the extensive technical skills required for qualified surgeons or other practitioners of technical specialties. However, these skills are included across most of the competencies and one goal of the ACGME initiative is for training programs to develop novel and accurate measures of these competencies in practice. In 2001, a survey confirmed that the vast majority of program directors in general surgery are in favor of surgical training outside the operating room to augment and complement hands-on experience with



Figure 2 The LapSim virtual reality system: The laparoscopic VLI interface with a PC-based desktop computer.

real patients[42]. The discrepancy between the demand for guidelines for laparoscopic simulator training for surgical residents and the application of these methods is telling. Curriculum development and validation of these methods is now of the utmost priority.

The first nationwide curriculum aiming to address this need is described in the FLS program[1]. This program is available for purchase by individual programs but is primarily designed as an independent evaluation tool. The FLS curriculum does not provide a mechanism for purchasing programs to internally evaluate resident performance or recommend distinct training guidelines. In addition, the FLS was deliberately designed to not include virtual reality computer simulators because of the acquisition costs involved and the lack of standardization and complete validation studies[1]. SAGES intends the FLS to become the standard for evaluation of basic laparoscopic skill acquisition but FLS may have limited long-term appeal if it does not incorporate virtual reality technology as it evolves.

To justify the steep acquisition costs, the validity and reliability of new teaching and evaluation tools must be supported by convincing studies before their widespread acceptance can be expected. Up until now, most skill evaluation for laparoscopic training and, in fact, surgery training in general, has been based on subjective evaluation and recommendation, a system that has been criticized for its lack of validity[43]. More recently simulation systems are being used in some training programs for the objective assessment of individual technical performance and as a supplement to the traditional evaluation[18,44-46]. An important aspect of a valid training system is that novices show an improvement of their skills over time. This has been demonstrated in multiple studies to be correct for the MISTELS, and more recently, for the VR-simulators[32,34,36,47]. Interestingly, some studies seem to support the work hours mandates. VR simulators have been used to demonstrate a decrease in psychomotor skills in residents subjected to sleep deprivation from a night of trauma call. Although the time to complete the tasks was unchanged, the number of errors was increased[48]. Another interesting study shows that alcohol intoxicated medical students perform worse in a VR simulator and regain their capabilities once sober[49]. The conclusions of these studies are quite serious and they rely on the

discriminative validity of the simulator used to assess differing performance.

Despite strong evidence supporting the use of surgical simulators in minimally invasive training of surgical residents, the learning curves to acquire psychomotor skills are relatively unknown within the field of surgical simulation. It is reasonable to think that "distributed practice" or frequent, repeated training provides longer term skill acquisition versus "massed practice", or intense short term training[50]. In a study evaluating the learning curve in laparoscopic skill training, different learning curves existed for surgeons with different laparoscopic backgrounds[24]. Much remains to be learned in determining the most efficient methods of expanding psychomotor skills. Contributing factors need to be identified and evaluated to determine the specific types of exercises that lead to improved performance in the operating room. The optimum practice time and requirements for continued practice need to be delineated.

MAINTENANCE OF SKILLS AND CREDENTIALING OF SURGEONS

As technologies and procedures evolve, newer techniques may require additional specialized skills. How do hospitals, practices, other physicians, and patients ensure that surgeons possess the requisite skills to perform these procedures safely and deal with intraoperative complications? How do we ensure the surgeons retain the skills to maintain a high level of practice? These questions surfaced before the advent of laparoscopic abdominal surgery and persist today with greater urgency because of the requirements and demands of modern practice[51]. The American Board of Surgery (ABS) is considering an objective skills proficiency examination to supplement the current knowledge-based and judgment assessments that comprise the surgery Qualifying and Certification exams. A completely objective, reproducible, and universally accepted evaluation tool is currently lacking, but it is likely that such a system will be developed and implemented in our practice lifetime. Similar to the aviation industry, a surgeon's skills at handling a difficult or emergent problem could be assessed. An exam could incorporate assessment of judgment and anatomic awareness. This exam could be employed for ongoing assessment of skill level at predefined intervals, much like the current recertification exam system. Patients entrust their lives to surgeons who rely on their judgment, visual and psychomotor skills. In this aspect, surgeons share common ground with airline pilots and it seems logical that surgeons, too, should be involved in an established method of acquiring and maintaining their high level technical skills. Surgical simulators will likely become an indispensable tool to these ends. The standards and performance expectations for such a system should be designed and validated by surgeons and surgeon-educators to ensure the exams are fair and reproducible.

The surgery of the future

Surgical simulators have the potential to be much more than tools for training and evaluation. As the technology continues to develop, the high standards required for appraisal and certification should allow future generations of simulators to also be used for operative planning. Complex operations will be planned and practiced in a virtual reality environment based on three dimensional imaging technologies imported into the device. This will be enabled by the same technology that allows us instant access to high-resolution computed tomography (CT) and magnetic resonance imaging (MRI) at our desktops today. After a surgeon has sufficiently practiced the virtual procedure, he would then perform the actual operation in the patient with significantly less operative morbidity, or perhaps with augmented-reality computer guidance[52]. The potential benefits to the patient are enormous.

In conclusion, surgical skills training is undergoing a dramatic transformation. Modern technology and training techniques, combined with external pressures and mandates are forcing surgeon-educators to rethink previously held principles. Simulators in their current form can facilitate the improvement of laparoscopic skills and operating room performance of surgical residents. Development of standardized training curricula for surgical residents remains an urgent and important agenda, particularly for minimal invasive surgery. An innovative and progressive approach, learning from the experiences of the field of aviation, can provide the foundation for the next century of surgical training. As the technology develops, the way we practice will continue to evolve, to the benefit of physicians and patients.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Management of diverticular disease is changing

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Abstract

Diverticular disease of the colon is primarily a disease of humans living in westernized and industrialized countries. Sixty percent of humans living in industrialized countries will develop colonic diverticula. It is rare before the age of 40, but more prone to complications when it occurs in the young. By age 80, over 65% of humans have colonic diverticula. The cause remains uncertain, but epidemiologic studies attribute it to dietary fiber deficiency. The cause of diverticulitis remains uncertain, but new observations and hypotheses suggest that it is due to chronic inflammation in the bowel wall. Standard medical therapies of bowel rest and antibiotics are still the recommended treatment. However, changing concepts and new therapies indicate that anti-inflammatory agents such as mesalamine and possibly probiotics may be helpful in shortening the course and perhaps preventing recurrences. Standard surgical treatment for perforation for severe acute disease has developed so that two-stage procedures are recommended. In addition, laparoscopic surgery has proven safe and may slowly become the technique of choice.

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Key words: Diverticulitis; Mesalamine; Treatments

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INTRODUCTION: EPIDEMIOLOGY AND PATHOPHYSIOLOGY

Diverticulosis of the colon is an acquired condition that results from herniation of the mucosa through defects in the muscle layer. Epidemiological and anatomic studies have revealed that diverticular formation of the colon is primarily a disease that occurs in industrialized and westernized countries[1,2]. Approximately 60 percent of humans over age 60 living in westernized countries will develop colonic diverticula[3]. Postmortem studies reveal that it is rare prior to age 40, but increases with age so that one can estimate that by age 80 approximately 65 percent of patients have diverticula of the colon[4,5]. Of major importance is that approximately 25%-30% of these patients will develop a complication: diverticulitis, bleeding, or perforation[5,6]. It is now widely accepted that chronic diverticular formation occurs in westernized societies due to a lack of fiber in the diet[6,7]. The assumption is that decreased dietary fiber intake results in decreased intestinal contents and, hence, decreased size of the lumen, which results in the transmission of muscular contraction pressure to the wall of the colon rather than to the contents of the lumen. The result of increased force (pressure) on the wall is the formation of diverticula at the weakest point in the wall: the sites of penetration by blood vessels.

The cause of acute diverticulitis remains obscure. It has been speculated that obstruction at the mouth of the diverticulum results in diverticulitis, similar to appendicitis, but this is no longer the accepted theory, and some feel that chronic inflammation precedes clinical diverticulitis[6]. Descriptions of diverticular disease may be divided into two stages: (1) diverticular formation; and (2) diverticulitis with either pericolitis (microabcess), pericolic phlegmon, pericolic abscess, pelvic or intraabdominal abscess, and free perforation leading to bowel obstruction, fistulization, or bacteremia and septicemia[8]. In trying to assess the first stage of the disease, our preliminary evaluations identified that there is a microscopic colitis associated with mucosa of the colon adjacent to the diverticula[9]. Others have also identified asymptomatic diverticular inflammation at screening colonoscopy[10].

As illustrated in Figure 1, we hypothesized that diverticular disease is formed in the setting of low fiber

diet. Our studies have revealed that Connecticut diets are low in fiber[11]. Furthermore, our laboratories and others have established that the indigenous bacterial flora in subjects eating a low fiber diet is decreased and altered as compared to subjects eating high fiber[12,13]. Because of this alteration, we postulate a very mild colitis may occur and may progress to a more focal, acute diverticulitis. The sigmoid colon has the smallest diameter of the colon and a different motility pattern. It is the most common site of diverticular formation in western countries. Post mortem studies[4] of 90 colons revealed that 26% had numerous diverticula limited to the sigmoid, but 15% also had an occasional diverticulum of the sigmoid, 30% had sigmoid and descending colon diverticula with 16% scattered throughout and 4% in the sigmoid, descending and transverse. The cecum and ascending colon make up less than 5%. However, right-sided diverticula are definitely more common in humans in Asia and Hawaii[6]. Spastic motility disturbances are associated with the most common forms of the disease in western society. The sigmoid may develop a corrugated appearance. Initial pathology studies suspected an increase in smooth muscle cells, but pathologic studies clearly reveal there is, in fact, an increased deposition of elastin between the myocytes[14].

INFLAMMATORY BOWEL DISEASE AND DIVERTICULAR DISEASE

The simultaneous occurrence of both inflammatory bowel disease and diverticulosis was first discussed by Schmidt and colleagues in 1968[15]. The overlap of Inflammatory bowel disease (IBD) and diverticular disease is being recorded more commonly[16]. Much debate still exists as to whether there is increased prevalence when the two phenomena occur together. However, there clearly is an overlap syndrome in which clinicians must differentiate acute diverticulitis from chronic Crohn's disease[16,17]. Makapugay and colleagues described a picture of chronic colitis associated with diverticular disease. Their work indicated that this may be more prevalent than suspected[18]. Peppercorn described eight patients who were more than 60 years of age and presented with segmental chronic colitis associated with diverticula[19]. Imperiali and associates performed a large perspective study to identify IBD-like lesions[20]. They reviewed 5457 consecutive colonoscopies and found that 733, or 13.5%, had diverticula, but only 20 of these patients (0.36%) met all of the endoscopic criteria for segmental colitis as well. They indicated that the phenomenon was less common than previously suspected.The picture is still not clear, however, since none of the studies did mucosal biopsies to indicate whether there was a low level of inflammation in mucosa adjacent to diverticula that is endoscopically normal, as indicated in some pilot studies[9,20]. Clearly, more studies are needed to fully evaluate the significance of the association of these two phenomena.

THE CLINICAL PICTURE AND DIFFERENTIAL DIAGNOSIS

When colonic diverticula are discovered during an examination such as barium enema, CT scan or colonoscopy which were done for screening or other reasons, they are assumed to be asymptomatic. Since the incidence is so high, most of these patients are not treated. However, as many as 5%-20% of patients with diverticula of the colon are symptomatic and can develop complications[21]. The clinical presentation depends on the degree and extent of the inflammation and the degree of loss of normal bowel function due to sigmoid diverticular formation. The most common presentation is that of left-lower quadrant pain associated with either mild constipation or diarrhea. When the inflammation is severe or there is a micro-abscess or abscess formation, the pain will be more severe and can present as an acute abdomen. Determining the total white blood count is helpful. It is usually elevated when the inflammation is severe. Depending on the severity, there may or may not be elevated temperature. If there is true perforation, the abdomen is much more tender with possible rebound, and a picture of peritonitis with a markedly elevated white blood count and perhaps a toxic-appearing patient. Because of the variable position of the colon and differing anatomy in different individuals, the pain might be located more on the right side or in the pelvis or even in the upper quadrants. When this occurs, a differential diagnosis between gynecologic disease or other diseases attributed to the abdomen must be considered. Radiologic findings are extremely helpful. Because of the possibility of perforation, a barium enema is no longer the preferred procedure. Computerized tomography (CT) scanning is now accepted as the most helpful diagnostic procedure. Areas of inflammation and the associated mesentery soft tissue are identifiable as well as abscesses. It must be kept in mind that it takes time for an abscess to form, and it might be missed on the initial CT examination. A study of 420 patients with left-sided diverticulitis proven at surgery compared CT to contrast enema. The CT was superior, was more sensitive, and was significantly better in grading the severity of inflammation[22]. CT has also proven most helpful in the management of complicated

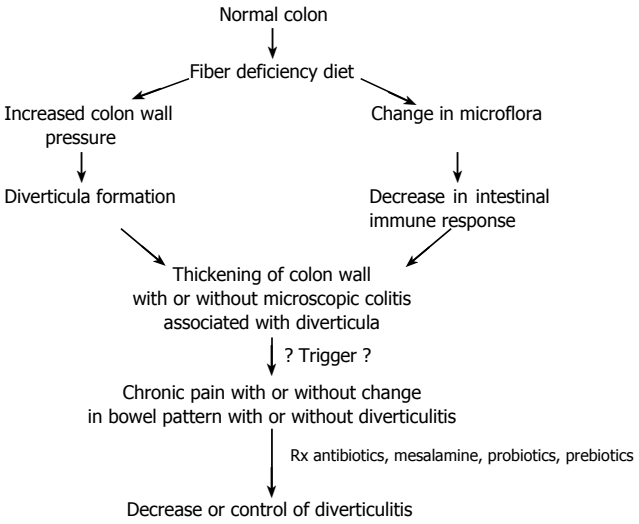


Figure 1 Pathophysiologic development of diverticulitis and proposed therapy.

diverticulitis. It is shown in correlated studies that once an abscess is present, there is a high risk of failure from non-operative management. However, when management is in question, CT is of great help in defining the course of treatment. Furthermore, CT can be very helpful in guiding percutaneous drainage of abscess in order to prevent or delay surgery in selective cases[23].

Colonoscopy is used judiciously when there are any suspicions of very acute inflammation. Once that has subsided, it can be helpful in excluding a suspected malignancy or overlap inflammatory bowel disease picture.

COMPLICATIONS

Perforations and diverticular hemorrhage are the major complications of diverticulosis. Perforation has to be treated acutely. However, when there is perforation into adjacent organs such as the bladder or vagina, appropriate gynecologic and urologic evaluations are necessary. Those patients are treated medically and then surgically if necessary. Diverticular hemorrhage is the most common cause of colonic bleeding in western societies. It is estimated that 3%-5% of patients with diverticula will have severe blood loss at some point. Diagnosis of the site of ongoing diverticular bleeding is important and may be difficult. Radio-isotope studies, colonoscopy, and arteriography, diagnostic and/or therapeutic may be helpful, but barium contrast enemas are of no help[21-24].

MEDICAL THERAPY

Simple, uncomplicated diverticular disease often requires no therapy. Many clinicians advise a high fiber diet, but the evidence that this is helpful in preventing diverticulitis is not yet clear. Once mild symptoms develop, clinicians may prescribe high fiber diets, plus anticholinergic drugs to theoretically relax the bowel. Dicyclomine is used most often. Once there is any sign of infection, such as elevated white blood count or low-grade temperature, antibiotics are indicated. Depending on the institution and the location, broad spectrum antibiotics are used since the colonic flora is a combination of aerobes and anaerobes. If symptoms are severe, combinations of ciprofloxin and gentamicin or metronidazole are recommended, or amoxicillin/clavulanic acid, or sulfamethoxazole/trimethoprim. Treatment is usually anywhere from 1-2 wk with careful monitoring and reevaluation. Diet is always of concern in these patients. When there are acute symptoms, the bowel is rested and low fiber diets are recommended. However, once recovery occurs then high fiber diets are recommended.

Medical treatment is changing. Based on the theory that there is chronic inflammation in the bowel wall, even between bouts of acute diverticulitis, enough interest was created to evaluate the use of anti-inflammatory agents. There are now four groups in Italy that have reported favorable results with the use of mesalamine alone or in combination with antibiotics. Evaluation of these studies[26-31] clearly reveals that mesalamine decreases the length of the course of the diverticulitis, improves management with the use of concomitant antibiotics, has a prolonged effect and also appears to decrease the incidence of recurrent attacks. From these studies, it appears that mesalamine is safe and holds promise as a new therapy in the treatment of acute and chronic diverticulitis.

The theory that inflammation is chronic in the formation of diverticulitis also raises the hypothesis that probiotics would stimulate the immune process and possibly be effective. Only one paper has appeared on this subject[32]. The authors compared the use of an anti-microbial agent plus an absorbent to the same regimen supplemented with non-pathogenic *Escherichia Coli*, Nissle strain. They found that the average remission for the antibiotic regime was 2.43 mo as compared to 14.1 mo for the antibiotic plus probiotic regimen. This is only one study, but the concept is valid and these findings indicate other studies are in order.

SURGICAL TREATMENT

Once medical treatment fails or when the presentation is one of acute abdominal perforation, surgery becomes essential. Standard surgical treatment today may include a longer period of observation, or percutaneous drainage of abscess, but may finally require surgical resection. Three-stage operations are no longer the treatment of choice. The two stage procedure where there is first a colostomy and then later closure of the colostomy after a 4-12 wk period of observation are preferred[21,24,33].

Most guidelines treating elective or non-urgent cases recommend that the resection must remove all thickened diseased colon, but not necessarily all of the proximal diverticulum-bearing colon. It may be acceptable to retain proximal diverticular colon as long as the remaining bowel is not hypertrophied[24]. In almost all cases, all of the sigmoid colon should be removed. In urgent and emergent cases, the resection and diversion are required. In rare cases, it may be attempted in a one-stage procedure.

With the advent of laparoscopic procedures, sigmoid resection may benefit from the approach[34]. Laparoscopic surgery for diverticular disease can be done safely with low conversion and complication rates when performed properly in select patients[34]. When diverticular hemorrhage does not stop, surgery may be indicated. At times it may be emergent. Segmental resection is certainly preferred, but hemicolectomy is often necessary. The operative mortality rates are high, between 9%-11%, and largely depend on co-morbid conditions and the age of the patient. When it is difficult to identify the exact site, morbidity and mortality rates may increase to as high as 35%. Fortunately, most cases of bleeding stop spontaneously, but when surgery is required, the site of bleeding must be identified. On the rare occasion when it cannot, then total colectomy becomes necessary.

Finally, diverticulitis is unusual in young people, especially those under 40. However, when it does occur in this age group, it appears to be virulent and as many as 2/3 of these patients require surgical intervention[21].

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Role of calcium and other trace elements in the gastrointestinal physiology

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Abstract

Calcium is an essential ion in both marine and terrestrial organisms, where it plays a crucial role in processes ranging from the formation and maintenance of the skeleton to the regulation of neuronal function. The Ca^{2+} balance is maintained by three organ systems, including the gastrointestinal tract, bone and kidney.

Since first being cloned in 1993 the Ca^{2+}-sensing receptor has been expressed along the entire gastrointestinal tract, until now the exact function is only partly elucidated. As of this date it still remains to be determined if the Ca^{2+}-sensing receptor is involved in calcium handling by the gastrointestinal tract. However, there are few studies showing physiological effects of the Ca^{2+}-sensing receptor on gastric acid secretion and fluid transport in the colon. In addition, polyamines and amino acids have been shown to activate the Ca^{2+}-sensing receptor and also act as allosteric modifiers to signal nutrient availability to intestinal epithelial cells. Activation of the colonic Ca^{2+}-sensing receptor can abrogate cyclic nucleotide-mediated fluid secretion suggesting a role of the receptor in modifying secretory diarrheas like cholera. For many cell types changes in extracellular Ca^{2+} concentration can switch the cellular behavior from proliferation to terminal differentiation or quiescence. As cancer remains predominantly a disease of disordered balance between proliferation, termination and apoptosis, disruption in the function of the Ca^{2+}-sensing receptor may contribute to the progression of neoplastic disease. Loss of the growth suppressing effects of elevated extracellular Ca^{2+} have been demonstrated in colon carcinoma, and have been correlated with changes in the level of CaSR expression.

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Key words: Calcium; Trace elements; Gastrointestinal physiology

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INTRODUCTION

The gastrointestinal tract is in charge of handling the complex issues of nutrient, electrolyte and fluid absorption and the secretion of excess electrolytes and fluids. Body calcium homeostasis is regulated by the PTH and vitamin D feedback loop; additionally calcium plays a key role in many other mechanisms like intracellular signaling, cell differentiation, bone metabolism, *etc*. After identifying the calcium sensing receptor in several different tissues many studies were undertaken to characterize the role of extracellular calcium as a first messenger and the receptor as a calcium sensor of the cell. Ca^{2+}-sensing receptor transcripts and/or protein are expressed in the gastrointestinal tracts of fish[1], birds[2], amphibia[3,4] and mammals[5-10] including the human[5,11,12]. Tracings of the expression of the receptor in the gastrointestinal tract shows that the receptor goes back in evolution at least as far as cartilaginous fish (elasmobranchs), e.g., the dogfish shark[1]. In both cartilaginous and bony fish, the Ca^{2+}-sensing receptor has been shown to be expressed on apical surfaces of stomach and intestine[1]. More recent evidence suggests that the Ca^{2+}-sensing receptor may have evolved in the early marine environment in order to support osmo-adaptation. This notion is supported by the more general expression of the Ca^{2+}-sensing receptor in many other tissues outside the gastrointestinal tract that are involved in mono-and divalent ion transport both into and out of fish that live in a seawater environment that is rich in divalent minerals and sodium chloride[1,13]. This theme of the Ca^{2+}-sensing receptor linking divalent and monovalent metabolism is echoed in mammals (e.g., effects of the receptor on fluid transport by the colon; discussed later in this review).

In the amphibian, *Necturus maculosus*, Ca^{2+}-sensing receptor expression was detected on the basal surface of gastric epithelial cells[3]. Wherease in contrast the frog stomach, shows expression of the Ca^{2+}-sensing receptor

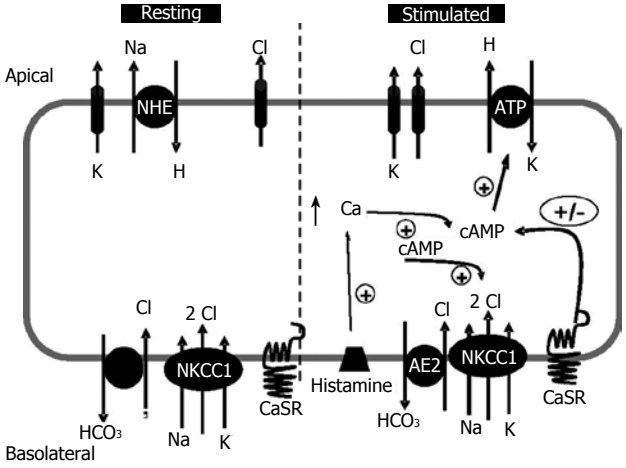


Figure 1 Simplified model of the parietal cell showing the current mechanisms involved in acid secretion in comparison to a resting cell. The Ca^{2+}-sensing receptor (CaSR) and the histamine receptor are located on the basolateral membrane. HCl secretion is mediated by H^+ extrusion *via* the H^+,K^+-ATPase coupled with Cl^- secretion *via* an apical channel. Activation of the CaSR causes acid secretion *via* the H^+, K^+-ATPase.

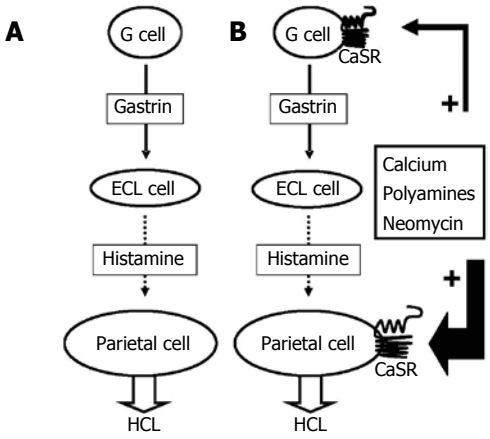


Figure 2 Schematic of acid secretion. **A**: Classically defined pathway of acid secretion: Gastrin released from the G cell binds to receptors on the ECL cell causing histamine release from the ECL cell. The histamine binds to receptors on the parietal cell and stimulates proton efflux; **B**: Modified model of acid secretion involving a functional CaSR on the basolateral membrane of both the G cell and parietal cell. Acid secretion can be activated directly by the CaSR on the parietal cell or *via* the G cell pathway.

on the apical membranes of acid-secreting oxyntic cells[4]. In the chicken, *Gallus domesticus*, the receptor was detected in the duodenum[2]. In mammals, a more complete exploration of Ca^{2+}-sensing receptor expression along the gastrointestinal tract has been performed[5-10,12]. Receptor transcripts and/or protein have been detected in: stomach, small intestinal, and colonic mucosal epithelia, as well as the underlying neural plexuses of Meissner and Auerbach. In addition, Ca^{2+}-sensing receptor expression has also been shown in several human intestinal cell lines (T84, HT-29, Caco-2, FET, SW480, MOSER and CBS;[8,14,15] in addition to primary cultures of human gastric mucosa and human parietal cells[11,16,17].

In mammalian stomach, the Ca^{2+}-sensing receptor has been identified on both apical and basolateral membranes of human G-cells (gastrin secreting cells;[16,17]) and mucous secreting cells[11] and on the basolateral membranes of parietal cells[4,6,18]. In small intestine, both apical and basolateral membranes of villus cells express the Ca^{2+}-sensing receptor[7]. In rat colon the receptor is expressed on both apical and basolateral membranes of surface and crypt epithelial cells[5,7]. A similar pattern of Ca^{2+}-sensing receptor immunostaining in rat was observed in both proximal and distal colon[5]. In the human large intestine, Ca^{2+}-sensing receptor has also been identified on both apical and basolateral membranes of crypts as well as in certain enteroendocrine cells at the base of crypts[7,12].

CALCIUM IN THE STOMACH

Overview

To produce the large quantities of 0.16 mol/L hydrochloric acid required for digestion of ingested food, the mammalian stomach has employed a complex series of neuronal, hormonal and/or paracrine[19]/autocrine feedback regulatory mechanisms[19-22] allowing for the continued production of acid. A model of acid secretion by the parietal cell is shown in Figure 1 summarizing data from many different studies[19,21-24]. Following stimulation, the H^+, K^+-ATPase (proton pump) is trafficked to the apical surface and is responsible for vectorial transport of protons[20-22,25-27]. Once at the membrane protons combine with secreted Cl^- ions to produce the concentrated acid. With the generation of a proton gradient enzymes such as pepsinogen are secreted into the lumen of the gland where they combine with the secreted acid and move from the gland into the interior of the stomach providing an effective solution that is capable of digesting proteins and processing them for amino acid or peptide reabsorption in the intestine.

The common model of acid secretion (Figures 1 and 2) involves stimulation of the gastric glands *via* either neuronal or hormonal pathways which results in the release of gastrin, which in turn acts directly on the endocrine (ECL) cells of the gland. Following stimulation ECL cells secrete histamine causing the parietal cell to insert proton pumps (H^+, K^+-ATPase) into the apical pole of the gland[28] which occurs *via* an active tubular-vesicular insertion mechanism that is used to transport H^+, K^+-ATPases into the apical region of the parietal cell[26]. In conjunction with pump insertion activation of an apical K^+ channel(s) provides K^+ ions that act as the counter ion and exchange with H^+ on the pump[20,21,23,29] (Figure 1) to sustain acid secretion. In addition to cation secretion an additional action of histamine release is to activate and/or insert Cl^- channels into the apical membranes of the parietal cells which mediate Cl^- secretion that accompanies H^+ secretion[20,30,31] (Figure 1). Thus the entire acid secretory process relies on: (1) insertion of H^+, K^+-ATPase into the apical surface for H^+ secretion; (2) concurrent activation of apical Cl^- channels mediating Cl^- secretion which allows for the formation of HCl; and (3) activation of apical K^+ channel(s) for K^+ recycling to the lumen of the gland and thereby providing the counter cation K^+ to maintain H^+, K^+-ATPase activity.

Loss of feedback control of acid (HCl) secretion in the stomach causes symptoms ranging from mild heartburn, to lesions and ulcerations of the gastric mucosa[19,32]. Left

untreated gastric ulcers can lead to abdominal bleeding, hyperplasia of cells, and potentially tumor formation. The complexity of the tissue due to the mixed collection of cell types, as well an additional layer of surface cells (secreting a protective mucous gel layer rich in bicarbonate) has prevented a complete analysis of glandular cell function and the associated feedback loops.

Role of the Ca^{2+}-sensing receptor in acid secretion

Following hormonal or neuronal stimulation there is a transient rise in intracellular Ca^{2+} concurrent with the onset of acid secretion in the gastric gland[19,20,22,33], this process has been associated with activation of pump translocation to the apical pole of the cell , and the associated secretion of acid[34]. Following this stimulatory period intracellular Ca^{2+} levels fall, and acid secretion diminishes to basal levels.

Activation of basolateral CaSR in *Necturus* gastric antrum by elevating extracellular Ca^{2+}, or using other receptor agonists like NPS-467, or neomycin resulted in a rapid hyperpolarization and a decrease in resistance of the basolateral membrane. Circuit analysis of these data suggested that these electrophysiological effects were due to activation of a basolateral K^+ channel(s)[3]. In rat stomach elevating extracellular Ca^{2+} leads to a rapid increase in intracellular Ca^{2+} in parietal cells which can occur in the absence of conventional extracellular secretagogues (e.g. histamine;[6]). To confirm that this rise in Ca^{2+} was associated with activation of the receptor, studies have been conducted using either the potent agonist Gd^{3+}, or addition of an allosteric modifier of the receptor such as the amino acid phenylalanine which both lead to an increase in the rate of acid secretion through the apical H^+, K^+-ATPase in the absence of secretagogues[18,35]. Inactivation of the Ca^{2+}-sensing receptor by reducing extracellular divalent minerals can also down regulate acid secretion even in the presence of potent secretagogues like histamine[18]. From the results of these studies it has now become apparent that the Ca^{2+}-sensing receptor (CaSR) plays an important regulatory role in acid secretion in mammalian gastric glands.

In addition to the animal studies on gastric glands, the CaSR has also been identified in human gastric tissues[11,16,17,36-38]. In the mucous epithelial cells, activation of the receptor results in a rapid rise in intracellular Ca^{2+} as well as a proliferative response when the cells were placed in culture[11]. In G cells, stimulation of the receptor results in gastrin release[16] accompanied by activation of phospholipase C[16] and an increase in intracellular Ca^{2+}[16,17]. The associated histamine release due to CaSR-mediated secretion of gastrin by G cells could account for rebound acid secretion that occurs following exposure to calcium containing antacids. All of these data are consistent with the scheme that the CaSR in the stomach could play an important role in both acid secretion and in mucosal repair. Activation of the receptor may act to modulate the rates of acid secretion in response to total body calcium homeostasis. Should there be a deficiency in calcium receptor activation would increase acid secretion, or prolong acid secretion, thereby allow maximal ionization of calcium from ingested foodstuffs and produce increased calcium delivery to the intestine. Increased intestinal calcium will activate the CaSR on the apical surface of the cells and result in inhibition of fluid secretion and enhanced absorption of the delivered Ca^{2+}. Over time as serum calcium rises, gastric CaSR would either become internalized, or deactivated, leading to a down regulation in acid secretion. In patients with Zollinger-Ellison syndrome (ZES; characterized by ulcer disease of the upper gastrointestinal tract, increased gastrin secretion, and non-β-cell tumors of the pancreas, i.e., gastrinomas), gastrin secretion and serum levels appear to correlate with the activity of the frequently associated hyperparathyroidism. This result would be consistent with gastrin secretion paralleling high PTH-driven elevations in plasma Ca^{2+}. Significant albeit variable, CaSR expression has been detected in human gastrinomas[37,38], suggesting that the receptor could mediate the effect of extracellular Ca^{2+} on gastrin secretion. Consistent with this explanation, activation of CaSR by raising extracellular Ca^{2+} increased rapidly intracellular Ca^{2+} that was not altered by the Ca^{2+} channel blocker, nifedipine[38].

CALCIUM IN THE INTESTINE

Overview

The Ca^{2+}-sensing receptor is expressed in epithelial cells along the entire small and large intestine, but only in colon has the receptor been studied in sufficient detail to permit comment on potential roles in normal intestinal function, in diarrheal states, and the effect of oral Ca^{2+} intake on reducing the risk of colon cancer. The expression of CaSR in nerve plexi involved in smooth muscle function and coordination, however, suggests a potential role in modulating intestinal motility. The latter could be important in coordinating food delivery (Ca^{2+}, amino acids, polyamines) and modulating intestinal motility to maximize nutrient absorption. In addition, an effect of CaSR activation on intestinal motility may be one factor contributing to the constipation that is associated with hypercalcemic states.

Calcium modulates fluid transport in the colon

The primary function of the colon is to both absorb and secrete fluid and thereby maintain normal salt and water homeostasis. The colon is a complex epithelium that consists of both extensive invaginations of the surface which are designated as crypts which make up approximately 90% of the epithelial mass and the remaining 10% being surface cells. Although earlier studies suggested that only surface cells absorb and only crypt cells secrete fluid into the lumen of the colon, recent evidence has established that both surface and crypt cells absorb and secrete fluid (see review for details;[39]). As over 90% of the colonic epithelial surface area is occupied by invaginations or crypts, these structures constitute the major functional unit of the colonic epithelium[40, 41].

The direction of net fluid transport is determined by the relative magnitudes of the absorptive and secretory fluxes. Under basal conditions (absence of hormones, drugs or other factors), net fluid transport by crypts is absorptive[42]. However, colonic crypts alter the direction

of net fluid transport to secretion upon exposure to cell permeable cyclic AMP analogues, (forskolin, or other agents that activate adenylate cyclase), or modulators of cyclic AMP metabolism such as phosphodiesterase (PDE) inhibitors[42]. Addition of cyclic AMP-generating hormones/factors like 5-hydroxytryptophan or prostaglandin E2 to the blood/interstitial surface of the crypt will also increases fluid secretion by colonic crypts[40,41]. Modulation of these fluid transport processes in the colon by cyclic AMP can result in profound fluid and electrolyte losses with associated secretory diarrheas, as is the case during cholera exposure (Figure 3)[40].

Some previous physiological studies in rats, measuring Ca^{2+} fluxes in isolated colonic mucosa suggested that the colon had the capacity to respond to changes in extracellular Ca^{2+}. For example, the colon, as is true for the small intestine, can absorb and secrete Ca^{2+} in response to changes in extracellular Ca^{2+} as well as in levels of 1, 25-dihydroxy vitamin D3[43-45]. These latter observations indicate that colonic mucosal epithelium *per se* is equipped with a Ca^{2+}-sensing mechanism. Recent studies have suggested that this divalent mineral sensing mechanism in colonic epithelia is the CaSR, based on immunolocalization of the receptor in apical and basolateral membranes and receptor function assays[3,8,9,12].

The activation of colonic CaSR by: Ca^{2+}, Gd^{3+} or neomycin, leads to rapid rises in intracellular Ca^{2+} in both surface and crypt cells[5]. The elevation in intracellular Ca^{2+} occurs within a few seconds, consistent with activation of the phosphatidylinositolphospholipase C-inositol 1, 4, 5-trisphosphate (PI-PLC-IP3) pathway by G protein-coupled cell membrane receptors. Ca^{2+}-sensing receptor-mediated increases in intracellular Ca^{2+} can be prevented by pre-treatment with U-73122, a specific inhibitor of phosphatidylinositolphospholipase C. This effect of PLC inhibition demonstrated that intracellular Ca^{2+} transients induced by Ca^{2+}-sensing receptor agonists were not the result of altered entry of extracellular Ca^{2+} into colonic epithelial cells but were rather due to receptor-mediated activation of PI-PLC. The receptor-mediated increase in intracellular Ca^{2+} concentration in colon was shown to be due to the release of Ca^{2+} from thapsigargin-sensitive cell stores[5].

The role of CaSR in modulating colonic fluid movement has been examined in isolated perfused colonic crypts using an in vitro micro-perfusion technique[5,46]. Under basal conditions (i.e., in the absence of forskolin, or other secretagogues), crypts exhibit net fluid absorption[47]. Following exposure to forskolin a net fluid secretion occurs[47]. Activation of either luminal or basolateral CaSR by Ca^{2+} and/or spermine reverses the forskolin-stimulated fluid secretion[5,46]. Further studies will be necessary to fully define the mechanism of CaSR effects on cyclic AMP-mediated fluid secretion. However, based on information recently obtained from the effects of CaSR activation on vasopressin-stimulated increases in cyclic AMP in the kidney thick ascending limb of Henle[48,49], we postulate CaSR-mediated elevation in intracellular Ca^{2+} would activate Ca^{2+}/calmodulin-sensitive phosphodiesterases that would metabolize intracellular cyclic AMP, and thereby abrogate fluid secretion (Figure 3).

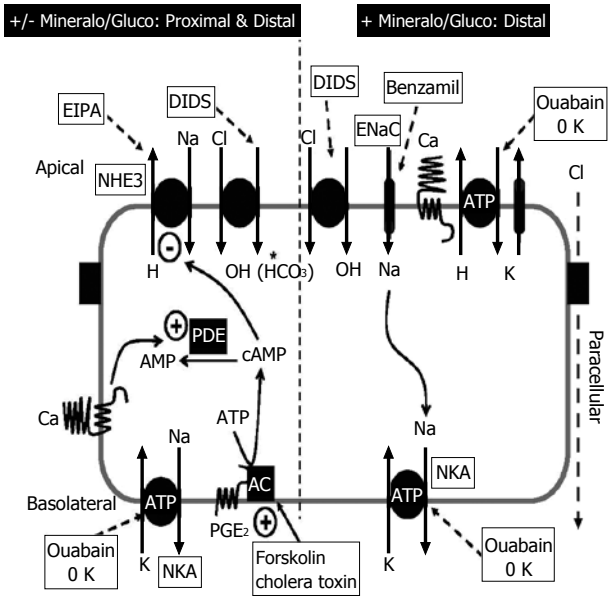


Figure 3 Cell model of proximal and distal colon. Shown are the known transport proteins in absence or presence of mineralocorticoid stimulation and their known inhibitors.

Increasing the levels of calcium on either the apical or basolateral membrane of the intact colon in Ussing chambers or in isolated perfused crypts leads to a decrease in fluid secretion. This decrease remains even in the presence of potent secretagogues such as forskolin; in fact there is enhanced absorption of fluid in the continued presence of potent secretagogues as long as the receptor remains activated[5,6]. In disease states or infectious states, fluid and electrolyte secretion can occur at pronounced levels and can cause dehydration and potentially death. By modulating the CaSR through increased delivery of calcium, or calcimimetic agents to the receptor, it appears possible that secretion could be stopped. This aspect of the CaSR could serve as an important new therapeutic target to modulate secretion and absorption of electrolytes along the intestine and combat secretory disease states.

POTENTIAL ROLES OF THE CaSR IN INTESTINAL EPITHELIAL CELL GROWTH AND DIFFERENTIATION AND NUTRIENT SENSING

Overview

The epithelium of the colon and the small intestine remains in a constant state of renewal. In the colon cells proliferate and become differentiated as they migrate from the base of the crypt towards the surface. Therefore cells at the base of the crypt are highly proliferative but less differentiated, whereas cells along the surface of the colon are highly differentiated and are in a non-proliferative state. Alterations of this tightly regulated process may lead to the development of hyperplastic events (polyps) and/or tumors. A potential role for the Ca^{2+}-sensing receptor in colonic epithelial cell proliferation, differentiation and development is suggested by the observations that receptor

activation reduces proliferation and induces differentiation of a variety of different cell types in addition to the intestinal epithelium. For example, activation of CaSR enhances cell differentiation in both mouse[50,51] and human[52,53] keratinocytes[54]. Moreover, activation of the Ca^{2+}-sensing receptor in other cells modulates proliferation and inhibits apoptosis[55-57].

The Ca^{2+}-sensing receptor responds not only to changes in divalent minerals but also to changes in organic nutrients (such as polyamines)[58] and amino acids[59,60]. Recently this effect was demonstrated in isolated rat gastric glands, whereby stimulation of the Ca^{2+}-sensing receptor by L-amino acids induced acid secretion in vitro[35]. In addition to activation of CaSR, there is evidence that certain amino acids can stimulate gastric acid secretion *via* the system L-amino acid transporter [Kirchhoff] which illustrates an increased layer of complexity in the process of acid secretion. Organic nutrients function primarily by altering the EC_{50} of the CaSR for Ca^{2+}, although direct agonist effects have been demonstrated. These nutrients could potentially alter the conformational structure of the Ca^{2+}-sensing receptor thereby enhancing the affinity for divalent ions and attenuating the cellular effects of receptor stimulation. The potential roles for these nutrients in coordinating protein and divalent mineral metabolism and in providing information on nutrient delivery to intestinal cells will be discussed in the following section.

Role in intestinal cell growth and differentiation

In small rodents such as rats and mice , dietary polyamine intake plays an essential role for normal gastrointestinal tract cell growth and development[61-65]. In humans, the postulated mechanisms for the pro-differentiation and anti-cancer effects of dietary Ca^{2+}/polyamines include: (1) formation of insoluble salts of Ca^{2+} with otherwise tumorigenic fatty acids and bile salts; and (2) modulation of the rates and/or fates of biologically active molecules such as nucleic acids, proteins and phospholipids[66-69]. The presence of CaSR on the plasma membranes of both surface and crypt epithelial cells raises the intriguing possibility that this receptor could mediate some of the dietary effects of Ca^{2+}, polyamines, and other nutrients on tissue modeling of intestinal epithelia.

Increases in polyamines, specifically spermine, results in the generation of IP3, raises intracellular Ca^{2+}, and modulates forskolin-stimulated fluid secretion, all consistent with activation of the colonic epithelial CaSR[46]. Polyamine (spermine > spermidine > putrescine)-mediated augmentation of intracellular IP3 and Ca^{2+} accumulation requires the presence of, and is potentiated by, extracellular Ca^{2+}. The EC_{50} for $Ca^{2+}{}_o$- mediated activation of the CaSR was also reduced by polyamines[46]. These results demonstrate that the colonic epithelial CaSR also positively responds to polyamines.

In cultured intestinal cell lines, CaSR has been shown to increase E-cadherin and reduce β-catenin production which are markers for intestinal differentiation[14,70,71]. In Caco-2 cells expressing the CaSR activation of this receptor by extracellular Ca^{2+} increases thymidine incorporation into DNA as a marker of cell proliferation[72]. Low concentrations of extracellular Ca^{2+} cause a PKC-dependent increase in c-*myc* protooncogene expression in Caco-2 cells and this pro-proliferative effect is abrogated by activation of the CaSR by increasing concentrations of extracellular Ca^{2+}[15]. The CaSR in keratinocytes and certain other cells has been shown to alter proliferation/differentiation and to modulate the activities of MAP and tyrosine kinases associated with cell proliferation[50-52,57,72-77]. All of these data, when taken together, support a potential role for the CaSR as a modulator of cell proliferation and differentiation in intestinal epithelial cells.

Role in colon cancer

Ingestion of high dietary Ca^{2+} promotes colonic mucosal epithelial cell differentiation, decreases cell growth, and reduces the risk for development of colorectal cancer (see recent summaries[78,79] and[14,15,77,80,81] and review by Karen Roland Cell Calcium special issue). Cancer of the colon and rectum is the second most frequently diagnosed malignancy in the United States in addition to being the second most common cause of cancer-related death (>56 000 American deaths this year). Observations that demonstrate that increases in dietary calcium reduce the risk of developing colon adenomas are noteworthy. Specifically, by increasing dietary calcium intake there has been: (1) a reduced risk for colorectal cancer by three-fold in men consuming 1400-1500 mg calcium per day, 19 year prospective study of men working at the Western Electric Co., Chicago[82]; (2) a significant reduction in colonic crypt cell proliferation and enhanced markers of cell differentiation in human subjects at increased risk for colon cancer[81,83,84]; (3) a reduced risk of colorectal adenomas in humans[85] also see[86-89]; (4) decrease in the incidence and number of carcinogen-induced colonic tumors in virtually all studies in rats, see[90] for a review; (5) significantly reduced recurrence of colorectal adenomas in a randomized, double-blind trial of 930 subjects[85]; (6) long term calcium supplements significantly suppressed colonic cell proliferation in adenoma patients[80]. Activation of the CaSR in human carcinoma cell lines by raising extracellular Ca^{2+} promotes E-cadherin expression while suppressing β-catenin activation[14], both markers of cell differentiation[70,71]. In addition, there has also been a correlation between CaSR expression and the stage of differentiation n human colon tumors[14]. These observations provide a significant body of evidence that increases in dietary Ca^{2+} reduce the risk of colon cancer and are mediated by activation of the CaSR.

Role of the receptor as a nutrient sensor

The ability of the CaSR to be activated by l-amino acids has been suggested as a link between protein and calcium metabolism[60]. This was suggested by the direct relationship between dietary protein intake and renal Ca^{2+} excretion[91,92]. A diet high in protein acutely increased urinary Ca^{2+} excretion[91,92] and a low protein intake induces elevated parathyroid hormone levels[93]. The increased urinary Ca^{2+} excretion associated with high protein intake appears due to elevations in intestinal Ca^{2+} absorption[91-93], although this has not been a universal finding[94]. As discussed in previous sections of this review, activation of the CaSR by Ca^{2+} stimulates gastric acid secretion, which

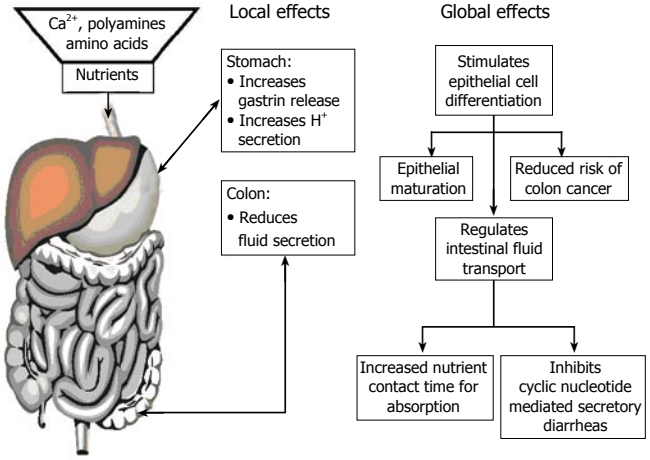


Figure 4 Current theory of roles for the Ca^{2+}-sensing receptor (CaSR) in the gastrointestinal tract.

in turn would promote acid digestion of proteins (together with peptidases). The release of l-amino acids would then promote Ca^{2+} absorption in small and large intestine by their synergistic activation of the Ca^{2+}-sensing receptor.

In summary, Figure 4 presents a current summary of the potential roles of the CaSR in gastrointestinal biology. Because of the unique properties of the CaSR in recognizing and responding to extracellular $Ca^{2+}{}_o$ and nutrients, this receptor presents a potential mechanism linking dietary metabolism (i.e., food digestion and nutrient absorption) to: (1) nutrient availability for epithelial growth and differentiation; and (2) protein and divalent mineral metabolism; (3) dietary Ca^{2+} intake and the associated reduction in risk of colon cancer; and (4) nutrient, salt and fluid homeostasis. In addition, the potential effects of nutrient activation of the CaSR on intestinal motility, coupled to the demonstrated reduction in fluid secretion, would increase nutrient-epithelial contact time and thereby enhance absorption. Finally, the potent ability of CaSR agonists to abrogate cyclic AMP-mediated fluid secretion by the colon has important implications for development of novel oral therapies of cyclic nucleotide dependent diarrheas like cholera.

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TOPIC HIGHLIGHT

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Updates on esophageal and gastric cancers

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Abstract

Esophageal and gastric cancers are both common and deadly. Patients present most often after disease progression and survival is therefore poor. Due to demographic variability and recent changes in disease incidence, much emphasis has been placed on studying risk factors for both esophageal and gastric cancers. However, with increasing understanding of these diseases, low survival rates persist and continued intensive studies are necessary to optimize treatment plans. This review article discusses updates in the evolving epidemiology, clinical presentation, risk factors, and diagnostic and treatment modalities of esophageal and gastric cancers.

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ESOPHAGEAL CANCER

Epidemiology

Overall, esophageal cancer contributes to 1% of all cancers diagnosed in the United States. Its prevalence in other geographical locations varies and higher incidences are seen in Asia, Africa, and Iran. However, the collective incidence continues to rise[1], and most of this increase is due to the increasing incidence of adenocarcinoma (AC). Today the incidence of SCC and AC are almost equal in the United States, where as of the 1960's, 90% of esophageal cancers were squamous cell (SCC). With this shift in histological type, there is also a shift in demographics. Both AC and SCC are seen more commonly in men, but SCC has a high prevalence in urban populations and in African-Americans, while AC is predominately a disease in Caucasians. Despite the evolving epidemiology of esophageal cancer and the development of new treatments there has been little change in survival. The five year survival for both AC and SCC remains approximately 14%[2].

Risk factors

Due to the demographic variability and the recent rise in AC incidence, much emphasis has been placed on studying risk factors for esophageal cancer. Currently smoking is a known risk factor in both SCC and AC and alcohol consumption is primarily a risk factor for SCC[3-5]. Dietary factors that have been more closely associated with SCC include foods with N-nitroso compounds, betel nut chewing, and hot foods and beverages. The development of SCC is also seen in patients with achalasia of the esophagus, with Plummer-Vinson syndrome, and with the autosomal dominate disease, tylosis. AC, on the other hand, has been closely link with the severe gastroesophageal reflux disease and Barrett's esophagus, the development of a columnar epithelium-lined esophagus[6-8]. Obesity, with a body mass index greater than 30, has also been shown to increase the risk of AC of the esophagus[9].

Clinical presentation

The most common complaints from patients with esophageal cancer are dysphagia and weight loss. Ninety percent of patients have difficulty swallowing solid food and subsequent changes in their diets lead to the loss of weight. Unfortunately the lumen obstruction that causes dysphagia is a sign of advanced disease. Patients with esophageal cancer often point to a specific trouble area in the neck or chest, unlike other dysphagia where there is generalized slowing of esophageal motility. Other less common symptoms include recurrent pneumonias secondary to aspiration, emesis of unprocessed food, hoarseness with recurrent laryngeal nerve invasion, or melena.

The physical exam unfortunately provides minimal additional information. Aside from weight loss, the physical findings are scarce except in metastatic disease where the liver may be enlarged or supraclavicular or

cervical nodes may be palpable. Laboratory values may demonstrate a low hematocrit secondary to bleeding, and most patients have elevated blood urea nitrogen and serum creatinine levels because of dehydration. Markers of nutrition, namely albumin, may also be low. Liver function tests should also be evaluated for metastatic disease.

Diagnostic modalities

Prognosis and treatment planning for esophageal cancer are dependent on the ability to make a diagnosis and establish a clinical stage. A barium swallow study is often done and is an appropriate modality for patient screening, especially in male patients over 50 years of age complaining of dysphagia. It provides cancer suspicion and tumor localization for later endoscopy. Esophagoscopy, however, is necessary for making a diagnosis. It allows for direct visualization and evaluation of Barrett's esophagus and tumors, and can provide a tissue diagnosis with a biopsy. Single biopsies of suspicious lesions in this setting are 93% accurate. The accuracy can improve to 100% with the addition of multiple biopsies (seven) and brushings[10]. An ultrasound probe combined with conventional endoscopy (endoscopic ultrasound) provides the most accurate estimate of disease stage by the tumor-node-metastasis criteria established by the American Joint Committee on Cancer. Ultrasound does so by evaluating the five layers of the esophageal wall with 85% accuracy and by detecting abnormal mediastinal lymph node metastasis with approximately 80% accuracy. EUS guided fine needle aspiration enhance this accuracy. EUS, however, is less accurate in accessing stage following the administration of preoperative chemoradiotherapy[11].

Metastatic disease is evaluated a number of ways. Computed tomography (CT) and positron emission tomography (PET) are both helpful in identifying distant metastasis. Bronchoscopy should also be performed in patients with upper and middle third tumors to exclude invasion into the trachea or bronchi. FNA of palpable supraclavicular or cervical nodes can provide additional valuable information about wide spread disease.

Surgical treatment

There are a number of different surgical approaches for treating early esophageal cancer. For the most part these procedures have similar perioperative mortality rates, approximately 4%[12,13]; however variations are seen in the types of complications that result from each given procedure. Choosing the best treatment strategy for an individual patient is therefore based on a number of different features of a specific lesion and on the preference and discretion of the surgeon. Important things to consider include the tumor location, the extent of lymphadenectomy necessary, and the use of neoadjuvant therapy. Resectable tumors of the middle and distal third of the esophagus are treated with a total esophagectomy. The two most common approaches are the transhiatal esophagectomy and the Ivor Lewis transthoracic esophagectomy. The primary differences between these approaches are the incision sites, the exposure provided by the incision, and the anastomotic site.

The transhiatal esophagectomy uses a midline laparotomy and a left neck incision. The laparotomy allows for blunt dissection of the thoracic esophagus and dissection and mobilization of the often conduit stomach. A cervical anastomosis is carried out through the left neck incision. The Ivor Lewis esophagectomy requires a midline laparotomy with a right thoracotomy. This allows for complete lymph node sampling under direct visualization and a high right sided intrathoracic anastomosis. A variation in this approach is a left thoracoabdominal incision (one incision), but some feel a high anastomosis in the left chest can present additional technical challenges. Another variation is a triincisional esophagectomy using a right posterolateral thoracotomy followed by a laparotomy and a left neck incision. Although the perioperative mortalities and the long term survivals are similar when comparing these different approaches, the anastomotic leak rate has been shown to be higher with the transhiatal approach, while respiratory and cardiovascular complications are more prevalent following the Ivor-Lewis approach[12-14]. However, cervical anastomoses provide a lower incidence of post-operative reflux, and anastomotic leaks are more easily controlled in the neck with lower associated mortalities.

The benefit provided from an extended, or three field, lymphadenectomy (mediastinal, abdominal, and cervical lymph nodes), through a transthoracic approach, remains controversial. A recently published prospective randomized trial from the Netherlands suggested a trend toward improved survival for 220 patients undergoing esophagectomies with extended lymphadenectomy for lower esophageal adenocarcinomas. Patients were randomly selected for either a transhiatal approach or a transthoracic approach with an extended lymphadenectomy. The overall 4.7 year survival rates were 29% and 39%, respectively[15], however, this was not a statistically significant difference. Currently in the United States this three field lymphadenectomy is not standard of care, although adequate lymph node sampling remains necessary for proper staging and prognosis.

Chemotherapy and radiation therapy

A survival advantage with chemoradiotherapy for esophageal cancer is still a matter of controversy. Currently neoadjuvant therapy seems to have a role in managing marginally resectable tumors and tumors with known lymph node involvement. Unpublished data from a trial in London, the MRC Adjuvant Gastric Infusional Chemotherapy Trial (MAGIC), presented at the American Society of Clinical Oncology this year, did show a statistically significant advantage in perioperative chemotherapy versus surgery alone in resectability, progression-free survival and overall survival for lower esophageal cancer[16]. Though this trial grouped esophageal and gastric Ge junction together, it is one of only a handful of studies to demonstate a significant survival advantage for neoadjuvant therapy, even though multiple trials have demonstrated the ability to downstage tumors and achieve a complete resection with clear margins. Though the evidence is sparse, neoadjuvant chemoradiation has

become the norm for patients with T3 or N1 disease on pre-operative evaluation. Definitive chemoradiotherapy is used alone in patients with unresectable tumors and post-operative chemoradiotherapy has shown modest survival benefits in small retrospective trials only[17,18] and is not generally recommended.

GASTRIC CANCER

Epidemiology

Gastric cancer is one of the most common cancers worldwide. In the United States alone, 21 860 patients have been diagnosed with gastric cancer this year, and 11 550 are expected to die as a result of this diagnosis[2]. Interestingly, its prevalence varies significantly with geographic location and in Japan, Korea, and parts of South and Central America, gastric cancer is up to 10 times more common than in the United States. Males are affected more often than females, and African Americans, Hispanics and Native Americans more than Caucasians. As our understanding of gastric cancer evolves, the epidemiology of the disease does as well. Since the 1930's there has been a 75% decrease in the overall incidence of gastric cancer in the United States[2], even without the implementation of routine screening modalities, like in Japan where the high incidence makes screening a cost effective modality. Simultaneous to the decreasing overall incidence is an increasing incidence of proximal gastric cancers and cancers of the gastroesophageal (GE) junction, escalating at a rate higher than that of nearly all other types of cancer. Proximal tumors are known to be more aggressive than distal tumors and have a worse prognosis for stage[20]. This shift to a more aggressive disease pattern and the inability to detect cancer at an early stage has limited our ability to make much progress in gastric cancer survival in the last 2 decades. The 5-year survival rate in the United States remains less than 30%[2].

The majority (95%) of gastric cancers are adenocarcinomas. Other tumors such as gastrointestinal stromal tumors, squamous cell carcinomas, gastric lymphoma, and carcinoid contribute to the other 5% of cancers. However the focus of this article will be on gastric adenocarcinomas.

Risk factors

Because of the geographic distribution and the recent shift in tumor anatomic location, much attention has been focused on determining risk factors associated with gastric cancer. Regional diets in particular have been under scrutiny to help shed light on causes of the disease. Studies comparing different foods have noted that western style breakfasts, diets high in antioxidants, and diets low in salt are shown to be inversely proportional to the incidence of gastric cancer[21]. With the discovery of the bacteria *Helicobacter pylor*i's (*H pylori*) role in ulcer disease, the role of *H pylori* in the development of gastric cancer has been examined as well. *H pylori* infection has now been show to increase the risk of body and antrum gastric cancers up to 6-fold[22,23]. In a prospective randomized control trial from China by Wong *et al*, there was a statistically significant decrease over 7.5 years in the incidence of gastric cancer in patients that received treatment for *H pylori* compared to those that did not receive treatment[24]. The same treatment, however, did not alter the incidence of gastric cancer in patients with premalignant lesions. There has also been an increase in the incidence of gastric cancer in patients with *H pylori* infection when the infection leads to gastric mucosal atrophy and pepsinogen 1 production compared to infected patients without this histological change[25].

A number of medical conditions have also been associated with gastric cancer development. The presence of adenomatous polyps increases the incidence of gastric cancers up to 20%, with greatest risk to patients with multiple polyps and polyps over 2 cm in diameter[26]. The same risk has not been seen in patients with hyperplastic polyps, which account for approximately 80% of diagnosed polyps. Chronic gastritis, pernicious anemia, intestinal metaplasia, acid hypersecretion conditions, Barrett's esophagus, and previous gastric surgeries have also been shown to influence the incidence of gastric cancer. Genetic risk factors for gastric caner include patients with hereditary non-polyposis colon cancer and familial polyposis coli. Cigarette smoking has also been linked with gastric cancer[27].

Clinical presentation

The vague clinical presentation of gastric cancer is the leading reason patients do not get diagnosed until the disease has progressed. Symptoms are nonspecific and are often not present. The most common complaint is epigastric pain, seen in 70% of early gastric cancers[20]. The pain is characterized as constant, nonradiating, and relieved sometimes with food ingestion or antacids. Less than 50% of patients with early cancers will complain of weight loss as a result of dietary changes secondary to the other symptoms. Other less common complaints include dysphagia, in patients with proximal cancers, and nausea and vomiting from gastric outlet obstruction in patients with distal tumors, anorexia, and melena.

The physical exam, unfortunately provides minimal additional information in early disease. Advanced disease may reveal a palpable epigastric mass, an enlarged liver, ascites, a periumbilical nodule or Sister Mary Joseph's node, left supraclavicular adenopathy or Virchow's node. An ovarian enlargement on pelvic exam (Krunkenberg tumor), or mass in the cul-de-sac on rectal exam (Blumer's shelf) may also be present. Elevation of serum alkaline phosphatase and anemia can be seen on laboratory values, but are not specific. Tumor markers are not yet a routinely used. Guaiac positive stools are seen in one third of patients.

Diagnostic modalities

A clear method for diagnosing and screening gastric cancer is important since its presentation often mimics other disease processes and there is a small window of time where the disease is potentially curable. Each different modality has its own strengths and weakness in diagnosing and staging the disease in order to plan the appropriate treatment. Although invasive and costly, endoscopy is

currently the most specific and sensitive method for obtaining a definitive diagnosis besides laparotomy, and has replaced barium contrast radiographs due to its ability to biopsy. It allows for direct visualization of lesions and the opportunity to obtain tissue samples. Endoscopy with seven biopsies of the margin and base of gastric ulcers has a sensitivity of 98% for diagnosing gastric cancers of all stages[10], compared to barium studies with a sensitivity of only 14% in early gastric cancers[28]. Barium continues to have a major role in diagnosing of linitis plastica, a diffuse type gastric cancer which is difficult to appreciate with endoscopy. Brush biopsies are performed in areas at risk of bleeding.

Staging for gastric cancer is based on the evaluation of the tumor (T), regional lymph nodes (N), and metastasis (M), as outlined by the American Joint Committee on Cancer system, and is essential for treatment planning and prognosis. Computed tomography (CT) is heavily relied on as a non-invasive test gathering information regarding metastasis and is performed routinely in patients diagnosed with gastric cancer. Although CT often underestimates nodal involvement and overestimates the depth of invasion, it is useful in assessing for liver metastases and distant spread. Endoscopic ultrasound, on the other hand, is more useful in evaluating the depth of tumor invasion (77% accurate) and regional lymph nodes (69% accurate)[29], but has no role in distant metastatic evaluation. Laparoscopy is the most invasive form of staging, but also 23% more accurate than CT scan in diagnosing peritoneal metastasis, which then precludes curative resection[30]. It is therefore reserved for patients with good performance status and those in which accurate staging is necessary for further treatment planning. New data also suggests that positive cytological washings at the time of laparoscopy are the most predictive preoperative factor of death from gastric cancer[31].

Surgical treatment

Complete surgical resection of gastric cancer provides the only chance for a cure. To obtain this goal, a 6 cm surgical margin with pathologic margins free from microscopic invasion should be attempted in order to offer the lowest possible rate for local recurrence. The type of gastrectomy performed to achieve this goal, however, has changed over time and still remains controversial. It was once thought that a total gastrectomy, with resection of splenic and celiac lymph nodes, splenectomy, and distal pancreatectomy was necessary for all gastric adenocarcinomas. The high morbidity of this operation, without a survival advantage over total gastrectomies with varying levels of lymph node dissections and partial gastrectomies, has led to a change from this practice. By evaluating a number of factors, including the tumor location, stage, and histological type, variation in surgical plans have been entertained and put into practice.

In proximal adenocarcinomas of the stomach, total gastrectomy or esophagogastrectomy remains the operation of choice for most surgeons. When compared to a proximal subtotal gastrectomy, a Roux-en-Y reconstruction has a lower incidence of reflux esophagitis[32]. In addition, the most common site of nodal metastasis for proximal tumors is on the lesser curvature. These nodes may be left behind in a proximal subtotal gastrectomy. However, a proximal gastrectomy, with an adequate node dissection, has an equivalent survival to total gastrectomy, and is still advocated by some surgeons. For distal tumors a subtotal gastrectomy is the procedure of choice. It has been shown that there is no added survival benefit for patients with distal tumors undergoing total gastrectomy and the operative morbidity and mortality is greater for patients who undergo total gastrectomy[33]. In addition, other studies have demonstrated that the quality of life is better for patients following subtotal gastrectomy compared to total gastrectomy[34,35].

Similar to esophageal cancer, the extent of lymph node dissection for curative surgery in gastric cancer remains controversial as well. Retrospective data from Japan has shown a survival benefit for patients with extensive lymph node dissection in stage II or stage III disease. The number of positive lymph nodes also predicts and influences survival when at least 15 lymph nodes, regardless of their position, are surgically biopsied[36,37]. However, extended lymph node dissections in the United States have been linked with increased operative mortality. Therefore numerous studies have looked specifically at this concept; the best way to limit operative mortality without leaving microscopic disease. The draining lymph nodes of the stomach are categorized into 16 stations. These stations are group into clusters referred to as D1, D2, and D3. D1 includes perigastric lymph nodes, D2 includes nodes along the hepatic, left gastric, celiac, and splenic arteries, and the splenic hilum, and D3 includes nodes within the porta hepatic and periaortic region. In the last 20 years, at least five prospective randomized trials have not show a statistically significant survival advantage of a D2 versus D1 lymphadenectomy or a D3 versus D2 lymphadenectomy, including trials from the Medical Research Council[38], the Dutch Gastric Cancer Group[39,40], and the Japan Clinical Oncology Group Study[41] in which 400, 711, and 523 patients were enrolled respectively. Criticism of these studies revolve around the inability of small sample sizes to adequately predict changes in the survival of patients with N2 disease since N2 patients only make up 30% of all the patients studied and only one-forth of these patients are predicted to survive following D2 dissections[42]. In addition, it was shown that surgeons varied in their compliance to dissection guidelines. Recent studies from the United States have demonstrated that in selected high volume centers operative mortality following D2 gastrectomies is no greater than that associated with the D1 resection[43]. Therefore, D2 resections have still been recommended by surgeons in the US who can perform the operation with low mortality. At minimum, a total of 15 lymph nodes must be removed at the time of gastrectomy to adequately stage patients[36] and this requirement has been adopted in the most recent AJCC staging handbook.

The role of laparoscopic surgery for gastric carcinoma is still in evolution. Currently there are a number of centers that successfully perform laparoscopic-assisted distal gastrectomies (LADG) and total gastrectomies, as well as hand-assisted gastrectomies with D1 lymph

node dissections for early gastric cancer treatment. Even more recently laparoscopic techniques have expanded to include treatment of cancers infiltrating deep submucosal layers and treatment of advanced tumors with no known lymph node metastasis with LADG and D2 lymph node dissections[44]. Although in general the operative time for laparoscopic procedures exceeds open procedures, outcomes of the laparoscopic approach suggest a decrease in postoperative pain and operative blood loss, shorter hospital stays, earlier return of bowel function, and fewer cardiac and respiratory complications[44,45]. Authors have not reported a statistically significant difference in the number of nodes dissected or in the adequacy of surgical margins when compared to open procedures. However these studies are limited by a low number of patients enrolled and a lack of long term follow-up regarding patient survival and the recurrence rates of cancer.

Chemotherapy and radiation therapy

Complete surgical resection remains the only chance for cure in patients with gastric cancer, but approximately 80% of patients with adequate negative margins still have recurrences and most patients ultimately die from their disease[46]. Therefore there has been a strong push toward developing effective chemotherapy and radiation regimens to be used with surgical management and many trials have been attempted or are underway. The results from these treatment modalities to date have been modest with little or no effect on overall survival. However in 2001, Macdonald et al looked at the survival benefits of chemoradiotherapy in 556 patients following curative gastric resections[47]. Results showed a 36 mo median overall survival with postoperative chemoradiotherapy compared to a 27 mo median overall survival with surgery alone. This data has established the current standard of care for patients in the United States who have undergone potentially curative gastric resections[47]. The regimen includes a combination of 5-fluorouacil (5-FU) (425 mg/m^2 of body-surface area per day) and leucovorin (20 mg/m^2 per day) for five days, followed one month later with radiation (1.8 Gy/d and 45 Gy total) and modified doses of 5-FU and leucovorin, followed by 2 more 5 d cycles of 5-FU and leucovorin one month later. New data presented from the MAGIC trial in London also demonstrated a significant improvement in resectability, progression-free and overall survival in patients with gastric and gastroesophageal junction tumors given perioperative chemotherapy compared to surgery alone[16]. Other treatment options for unresectable tumors and for patients with distant metastasis are still being investigated in a number of ongoing clinical trials.

In summary, the epidemiology of both esophageal and gastric cancer has changed in the last few decades. Both the surgical management and chemoradiotherapy has evolved as well. Even with our advances in understanding these disease processes and with our improvement in treatment strategies, the overall 5 year survival rate remains exceedingly low and most patients die from their disease. This emphasizes the importance of continued research efforts into both esophageal and gastric cancer in order to continue to improve on what is known.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Update in management of mesenteric ischemia

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Abstract

Mesenteric ischemia disorders are precipitated by a circulation insufficiency event that deprives one or several abdominal organs of adequate respiration to meet metabolic demands. Although mesenteric ischemia occurs infrequently, the mortality rate is from 60% to 100%, depending on the source of obstruction. The successful outcome is dependent upon a high index of suspicion and prompt management. We briefly review the pathophysiology and presentation of the various ischemic entities and review the current state of the art in diagnosis and treatment. Despite advances in both diagnosis and treatment, prompt diagnosis and supportive care remain critical for successful outcome. New imaging techniques, endovascular therapy and emerging research may improve our approach to this deadly condition.

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Key words: Mesenteric ischemia; Duplex ultrasonography; Plasma D-dimer; Computed tomography

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INTRODUCTION

Mesenteric ischemia disorders are precipitated by a circulation insufficiency event that deprives one or several abdominal organs of adequate respiration to meet metabolic demands. Although mesenteric ischemia occurs infrequently, the mortality rate is from 60% to 100%, depending on the source of obstruction[1]. Even with recent technological advancement, successful outcome is dependent upon a high index of suspicion and prompt management.

This spectrum of disorders can be roughly categorized by the acuity or chronicity of presentation. Acute mesenteric ischemia is further subdivided based on the etiology of the occlusion; embolic, thrombotic or nonocclusive. It has been estimated that one third of acute cases are caused by arterial embolism, one third caused by acute arterial thrombosis, and the remaining majority of acute cases are caused by a nonocclusive etiology, with a small proportion of cases from venous thrombotic etiology[2,3]. Chronic ischemic symptoms usually stem from longstanding atherosclerotic disease of two or more mesenteric vessels. This article will briefly review the pathophysiology and presentation of the various ischemic entities and review the current state of the art in diagnosis and treatment.

PATHOGENESIS AND PRESENTATION

The classic presentation of patients with embolic disease is of sudden catastrophic abdominal pain out of proportion to physical examination findings. Only one third of patients present with the classic triad of abdominal pain, fever and heme-positive stools. These patients often have a history of cardiac disease which might predispose to embolus formation (atrial fibrillation, ventricular aneurysm, mitral valve disease, *etc.*). If there is a thrombotic component to the acute event, a history of chronic abdominal pain and coexistent atherosclerotic disease can be elicited. Venous thrombosis of the visceral vessels can also precipitate an acute ischemic event. Compromised venous return leads to interstitial swelling in the bowel wall, with subsequent arterial flow impedance and eventual necrosis. Etiologic factors for venous thrombosis include portal hypertension, intrabdominal sepsis, cirrhosis, pancreatitis, malignancy and trauma. Less common causes of acute mesenteric ischemia include arteritis, ergot administration, aortic dissection with concomitant occlusion of visceral vessels, and iatrogenic causes related to cardiopulmonary shunting in cardiac surgery.

Patients who present with nonocclusive mesenteric ischemia do so in a clinical setting of extremely low-flow splanchnic circulation, usually in the presence of vasopressor use. No vascular occlusion is usually demonstrated because pulsatile blood flow is present in larger arteries[4]. Patients with severe cardiac failure are at risk for nonocclusive mesenteric ischemia from vasospasm related to elevated sympathetic activity or hypovolemia.

Catecholamines and medications such as digitalis, by interfering with the autoregulation of mesenteric circulation, can also cause vasospasm.

The etiology of chronic ischemia is also multifactorial and many of the causes are similar to those listed for acute ischemia, but rarely from emboli. The most common cause of chronic intestinal ischemia is atherosclerotic occlusion or severe stenosis of the mesenteric arteries. A stenosis of > 50% is present in 18% of patients older than 65 years, but very few of these patients have symptoms[5]. Slow-growing obstructions are usually the source of this disease. Similar to acute disease, chronic nonatheromatous lesions which probably occur in a higher proportion in the visceral vessels include Takayasu's arteritis, dysplastic lesions, and Buerger's disease[6]. Chronic symptoms are caused by the gradual reduction in blood flow to the intestine. Since total blood flow to the intestine can vary from 20% when fasting to 35% after eating, symptoms occur with the demand for blood flow[7]. The three major gastrointestinal arteries that arise from the abdominal aorta are the celiac axis, the superior mesenteric artery (SMA), and the inferior mesenteric artery (IMA). Since collateral circulation is present at several levels, patients may be asymptomatic even with stenoses in several arteries. When SMA is occluded, the pancreaticoduodenal arteries supply blood via the hepatic and gastroduodenal arteries to the bowel, and when the celiac artery is also occluded, IMA supplies blood to the small bowel via the left colic branch. Frequently angiography will demonstrate a large meandering mesenteric artery, which is an important vessel in the collateral circulation from the inferior mesenteric artery. Symptoms usually occur if two or more vessels are occluded. Chronic mesenteric arterial ischemia typically causes postprandial abdominal pain and weight loss. As the obstructive process progresses, chronic dull pain ensues. Chronic venous occlusion causes vague abdominal pain or distention and usually does not infarct the bowel. Frequently, it is found incidentally on abdominal imaging.

Adequate intestinal perfusion can usually be maintained at near normotensive blood pressures when only one artery is involved, and under these circumstances mesenteric ischemia rarely occurs. When multiple severe occlusions are present, acute intestinal ischemia occurs from the critical reduction in the blood flow. Severe symptoms and possible infarction of the intestines can occur. The bowel becomes ischemic when the intestinal blood pressure falls below 40 mmHg[7].

At the cellular level, ischemia causes mitochondrial dysfunction, loss of ion transfer regulation and intracellular acidosis. Changes in membrane permeability and release of free radicals and degradative enzymes lead to cell death and tissue necrosis. Experimental models in rats have demonstrated mucosal injury and bacterial translocation within 2 h of a 30 min hypovolemic, hypotensive episode[8].

DIAGNOSTIC MODALITIES

Over the last decade, duplex ultrasonography has shown promising results in the diagnosis of chronic mesenteric ischemia. Multiple studies, using angiography as a standard reference, have found that duplex ultrasonography is an accurate screening test for proximal stenosis or occlusion. End diastolic velocity greater than 45 cm/s or peak systolic velocity greater than 275 cm/s portends high specificity for SMA stenosis[9-11]. However, these findings must be correlated with a clinical scenario and angiographic findings. In addition, duplex exams are limited in quality by patient body habitus, overlying bowel gas, patient compliance, and operator dependence[12].

Magnetic resonance angiography, although initially criticized for limitations in resolution and oversensitivity, is being investigated for diagnosis of mesenteric ischemia. Recent studies show a disease specificity of 95%[13]. Li *et al*[14], used magnetic resonance oximetry to evaluate the percentage of oxygenated hemoglobin in the preprandial and postprandial SMA in patients with chronic ischemic symptoms. Compared to asymptomatic atherosclerotic patients, this study showed a statistically significant increase in oxygen extraction and a decrease in oxygenated hemoglobin in the mesenteric ischemia group. Combined with magnetic resonance angiography, oximetry may hold promise as a powerful diagnostic modality. Selective mesenteric angiography is considered to be the gold standard for the diagnosis of acute arterial occlusion. Abrupt cutoff of the SMA with the absence of collateral circulation is diagnostic with virtually 100% sensitivity in acute embolic occlusion[15]. The more important outcome of arterial catheterization in this era however, as discussed below, will be the development of endovascular therapy.

While imaging of the visceral vessels and involved organs are essential to determine severity of disease, physiologic or biochemical means of detection, both for diagnosis and as a predictor of behavior, do not exist. Serum lactate, an established marker of cell hypoxia, has been shown to have a sensitivity of 96% in patients with mesenteric ischemia[16]. However, lactic acidosis is often a late finding in the diagnostic pathway with concomitant shock, bowel necrosis and circulatory collapse. In this regard, several areas of research should be mentioned. Small animal models have shown that tachyarrythmias detected by electrophysiologic sensors of the bowel wall are sensitive indicators of visceral hypoperfusion. Seidel showed that transabdominally placed superconducting quantum interference devices can accurately detect small bowel electrical rhythms *in situ*. Moreover, in this animal model of SMA occlusion, the ischemic intestine exhibited decreased electrical activity which was detected with a sensitivity and specificity of 94% and 100%, respectively. This noninvasive modality stands to be validated with future work[17,18].

Recently, plasma D-dimer has been suggested as an early marker of acute ischemia. In animal studies D-dimer has been shown to correlate with the onset of ischemia and it functions as a time sensitive indicator of disease progression[19,20]. Similarly, the enzyme alcohol dehydrogenase has been identified as a potentially sensitive indicator of bowel ischemia as opposed to generalized systemic hypoperfusion[21]. Glutathione S-transferase, a detoxifying cytosolic enzyme, is also a substance of interest. It is released with cell membrane damage and several isoforms have been correlated with bowel specificity. This enzyme has also been shown to exhibit

a time and duration specific detectable increase with progressive tissue ischemia[22].

Computed tomography (CT) is a fast, widely available noninvasive modality that has improved enormously in recent years. In 2000, the American Gastrointestinal Association concluded that CT was of limited use in the diagnosis of acute mesenteric ischemia, except in patients suspected of having superior mesenteric vein thrombosis[15]. Since then, multidetector row computed tomography has emerged as the gold standard for evaluation of mesenteric ischemia. In one prospective series, the positive and negative predictive values in a series of 291 patients were found to be 90% and 98%, respectively[23]. In another prospective series of 62 patients evaluated for mesenteric ischemia with multidetector CT and mesenteric angiography, the sensitivity and specificity was found to be 96% and 94%, respectively[24].

TREATMENT OPTIONS

Management of mesenteric ischemia clearly depends on the nature, acuity and severity of disease. Early intervention involves resection of nonviable bowel, restoration of blood flow to the ischemic intestine and supportive care. In the acute thromboembolic event, operative embolectomy has been the traditional procedure with reasonable short and long term outcomes. Surgical revascularization for visceral ischemia due to an occluded SMA was first described by Shaw *et al*[25] in 1958, in which they reported two successful cases of mesenteric thromboendarterectomy. For chronic occlusive disease, when revascularization is considered, single-vessel bypass to the superior mesenteric artery has been very successful, even in patients with multiple-vessel occlusions. Cunningham *et al*, reported an 86% symptom-free rate at 5 years after surgical revascularization[26]. In one study, the operative procedure had a perioperative mortality rate of 3%, a 5-year survival rate of 61%, and a 9-year assisted primary graft patency of 79%[27]. Those who advocate multiple vessel revascularization suggest that there is a higher incidence of graft failure and recurrence of symptoms after single-vessel revascularization. At the Mayo Clinic, the 5-year graft patency rates have been 90%, 54%, and 0% with three-vessel, two-vessel, and one-vessel bypasses respectively[28].

Antegrade aortoceliac bypass and transaortic endarterectomy have been successful for poor-risk patients, and are usually adequate for multiple outflows. Antegrade mesenteric bypass grafts from the distal thoracic aorta have also been associated with low mortality and morbidity rates[29]. Antegrade bypass from the supraceliac aorta can reduce kinking, compression, turbulence, and thrombosis, but has been associated with renal ischemia and can be technically challenging. Retrograde bypass from the infrarenal aorta or iliac artery is technically easier and avoids renal ischemia, but has been related to lower inflow and graft kinking. Revascularization of the superior mesenteric artery and celiac axis has been used to minimize the recurrence of symptoms and organ infarction if one graft fails[30]. Graft failure has been reported to be higher in males[31]. While most patients with chronic venous mesenteric ischemia can be treated with anticoagulation therapy, the source of the thrombus must be determined and treated.

Since the first reports of percutaneous transluminal angioplasty of the visceral vessels in 1980, endovascular therapy for atherosclerotic disease has rapidly expanded in both scope and indication in the last ten years. In both the acute and chronic presentation of intestinal ischemia, endoluminal therapy has emerged as a main, if not first-line therapy. Symptom relief with implantation of stents in the proximal celiac artery has been reported[32,33]. In addition, the thrombosis rate of mesenteric prostheses has been reduced from 18% to 1% with the addition of antiplatelet therapy.

Thrombolytic therapy in the management of intestinal ischemia was first reported in 1979 with the successful application of intraarterial streptokinase in the SMA[34]. In those patients with ostial or short segment occlusion of the SMA and celiac artery, recanalization with or without concomitant fibrinolysis (depending on acuity and presence of thrombus) has been shown to have promising initial results[35]. In many centers, intracatheter fibrinolysis with or without embolus retrieval is the initial treatment for SMA thromboembolism[36-38]. With the increase in percutaneous approaches to therapy, concern has arisen regarding the durability of treatment. Hallisey *et al*, reported a 75% primary patency rate at a mean follow up of 2.3 years[39]. Matsumura *et al*, reported a 29% recurrence rate with endovascular treatment within 1 year of treatment[40]. It has been suggested that in selected patients, surgery should be the treatment of choice, because it provides better long-term primary patency[41-43].

When the results of open surgery are compared to those of percutaneous angioplasty and stenting, there is a higher incidence of recurrent symptoms after percutaneous angioplasty[44]. In a recent retrospective review, the Dartmouth group noted an increased rate of restenosis and reintervention in the stent group versus an historical control surgical group. However, despite reintervention, 93% were symptom-free at last follow-up with a significantly smaller periprocedural mortality rate compared to open surgical patients. In addition, the authors pointed out that in one case, stent placement allowed sufficient nutritional optimization as to allow a previously poor risk patient to tolerate a definitive open procedure[45]. Clearly, there may be a role for minimally invasive therapy in high risk patients. With the accumulating experience in high volume endovascular centers, emerging prospective data may be able to more adequately analyze outcome data between the two treatment modalities.

In the subset of patients who suffer from nonocclusive ischemia, intraarterial vasodilator therapy has been largely responsible for the decrease in mortality from 70% in the 1980's to 50%-55% during the last decade[46]. Papaverine, the most common agent in practice today, is a nonaddictive opium derivative extracted from the poppy plant. It functions as a phosphodiesterase inhibitor and is usually infused directly into the SMA at a rate of 30 to 60 mg/h. The resultant accumulation of cyclic adenosine monophosphate acts to relax vascular smooth muscle. Recent work has shown that iloprost, a synthetic carbacyclin derivative of epoprostenol may improve SMA

flow in a hypotensive porcine model. Iloprost, a potent inhibitor of platelet aggregation and powerful fibrinolytic agent, increases flow with no appreciable change in mean arterial pressure and cardiac output. This suggests a possible role for prostacyclin analogues in the treatment of nonocclusive ischemia[47].

Investigational therapy in the treatment of mesenteric ischemia has focused on pharmacologic strategies to treat mesenteric ischemia. These include renin-angiotensin blockers and free radical inhibitors. The renin-angiotensin axis has been shown in a porcine model to play a prominent role in vasoconstriction. In this model, blockade of this signal pathway was able to prevent the ischemic injury from induced splanchnic vasoconstriction[48]. These findings imply that hemodynamic forces of nonocclusive ischemia are mediated, at least in part, by the renin-angiotensin axis.

The dual-hit hypothesis of ischemic injury is comprised of the initial hypoxic episode and then the subsequent reperfusion injury after reestablishment of forward flow. There is significant data to suggest that reactive oxygen metabolites such as superoxide, hydrogen peroxide, and hydroxyl radicals mediate ischemic injury. These substances are thought to activate phospholipase A_2, which leads to chemotaxis and leukocyte-mediated vessel injury[49]. In this regard, inhibition of xanthine oxidase by allopurinol has been shown to attenuate the epithelial cell damage during reperfusion[50]. Similarly, pretreatment with superoxide dismutase and dimethyl sulfoxide attenuates reperfusion injury in animal models[51-53]. However, since most of these therapies require pretreatment to be effective, it is difficult to foresee clinical applicability at this time.

In conclusion, mesenteric ischemia, a spectrum of disorders with multiple etiologies still carries significant morbidity and mortality. Despite advances in both diagnosis and treatment, prompt diagnosis and supportive care remain critical for successful outcome. New imaging techniques, endovascular therapy and emerging research may improve our approach to this deadly condition.

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TOPIC HIGHLIGHT

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Surgical perspectives in peptic ulcer disease and gastritis

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Abstract

For much of the twentieth century, surgery was frequently the solution for peptic ulcer disease. Our understanding of the pathophysiology of ulcers paralleled the development of potent pharmaceutical therapy. As the surgical world developed parietal cell vagotomy which would minimize the complications of surgery, patients failing medical therapy became rare. Emergent surgery for complicated peptic ulcers has not declined however. The development of proton pump inhibitors and the full understanding of the impact of *H pylori* has led to a trend towards minimalism in surgical therapy for complicated peptic ulcer disease. In addition to the changes in patient care, these developments have had an impact on the training of surgeons. This article outlines these trends and developments.

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Key words: Perspectives; Peptic ulcer disease; Gastritis

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INTRODUCTION

During the twentieth century there were major advances in the understanding of the pathophysiology of what were known as acid-peptic diseases. These advances included the understanding of the role of the vagus nerve, the discovery and eventual peptide sequencing of the hormone gastrin and the landmark discovery of *H pylori*. Advances also occurred in medical therapy. Selective histamine receptor blockers and proton pump inhibitors were developed. Antibiotic therapy was found to eradicate *H pylori*. Thus changes in treatment of acid-peptic disorders have paralleled the changes in the understanding of the pathophysiology. This review will cover current perspectives in surgical treatment and how advances in medical management have impacted surgical practice.

GASTRITIS

Gastritis literally means inflammation of the stomach. This term may refer to acute gastritis or chronic gastritis, two very different disease entities. The Sydney System, used by pathologists and gastroenterologists to classify gastritis, emphasizes the importance of combining topographical, morphological, and etiological information into a schema that helps to generate reproducible and clinically useful diagnoses[1].

Acute gastritis is in many cases synonymous with erosive gastritis. This may be due to toxic damage to the gastric mucosa, commonly from non-steroidal anti-inflammatory drugs (NSAIDs) or alcohol ingestion. Erosive gastritis may also occur in critically ill patients with sepsis, shock or major body burns. On endoscopy, these patients have diffuse superficial gastric erosions and/or petechiae[2]. On histology, there is surface cell damage but a paucity of inflammatory cells. Supportive care with fluid resuscitation and anti-acid therapy results in rapid resolution of this type of gastritis in the vast majority of cases.

Historically, the first line treatment of acute erosive gastritis has been medical therapy. The first step is cessation of the insult, including stopping NSAIDs and resolving hypotension and/or hypoxia with appropriate supportive therapy. Therapy includes anti-acid therapy of any type to bring the gastric juice to a pH > 5 in all patients. In recalcitrant cases, additional treatment such as intravenous pitressin or octreotide may be required. Prior to 1980 there were failures of medical therapy, which required surgery for unremitting hemorrhage. Proposed operative therapy included anti-ulcer operations plus over sewing of bleeding sites, gastric resection and even gastric devascularization. The mortality of these operations ranged up to 40%[2]. Fortunately with modern care the need for surgery in these cases is beyond rare.

Chronic gastritis is most commonly due to *H pylori* infection. Most of these cases are asymptomatic and there

is poor correlation between symptoms and chronic *H pylori* infections with chronic inflammation. Again, surgery plays no role in these cases.

Another type of chronic gastritis is duodenal reflux or bile reflux gastritits. These cases may be spontaneous or secondary to prior gastric surgery. The cases are characterized by abdominal pain and bilious vomiting. On endoscopy, these patients have bile in the stomach, erythematous stripes or patches and sometimes mucosal edema[4]. On histologic examination, there is foveolar hyperplasia, mucosal edema and a paucity of inflammatory cells. Treatment with mucosal protective agents, prokinetic agents, and binders of bile salts has been disappointing. Surgical therapy consists of diversion of bile away from the stomach. In cases of spontaneous bile reflux gastritis, Madura has achieved excellent results with Roux-en-y choledochojejunostomy[5]. In the cases of secondary reflux gastritis (after gastric surgery) diversion of bile and duodenal contents away from the stomach has frequently been accomplished by Roux-en-y gastroenterostomy, with a long Roux limb (> 50 cm).

PEPTIC ULCER SURGERY

The surgical treatment of peptic ulcer disease, the main focus of this review, has changed dramatically over the years. To better understand the evolution of peptic ulcer surgery we must trace its development. We will review some of the highlights in the development of ulcer surgery as we know it today. In 1882 Czerny, a member of Billroth's department, performed local excision of a gastric ulcer, just one year after Billroth's first successful gastric resection for an obstructing carcinoma[6]. The Heinecke-Mikulicz pyloroplasty was first introduced separately by Heinecke in 1886 and by von Mikulicz in 1887. This commonly used pyloroplasty involves a longitudinal incision over the pylorus that is closed transversely. The Jaboulay pyloroplasty, introduced in 1892, is a side-to-side gastroduodenostomy used to bypass the pylorus. Finney later modified the Jaboulay pyloroplasty in 1902 by incising through the pylorus and creating a single lumen. It is important to recall that these operations were developed to treat ulcer induced fibrotic strictures.

Thanks to Pavlov's research, the role of the vagus nerves in gastric secretion was recognized by surgeons fairly early in the twentieth century. In fact, Laterjet *et al* appreciated the need for gastrojejunostomy for the gastric atony induced by vagotomy. Dragstedt popularized truncal vagotomy for the treatment of peptic ulcer disease during the 1940's. He published "Vagotomy for Gastroduodenal Ulcer" in the Annals of Surgery in 1945[7]. Dragstedt rediscovered that vagotomy alone resulted in gastric emptying problems, which again led to the combination of truncal vagotomy with a gastric emptying procedure. In 1937, Graham[8] described the omental patch that we so frequently call the "Graham patch" as a simple technique for treatment of a perforated ulcer.

In 1977, Nyhus *et al*[9] published a large cooperative prospective randomized study at the Veterans Administrations Hospitals showing the best results with vagotomy and antrectomy, making it the most frequently performed operation for duodenal ulcers during the 1970's and 1980's. The truncal vagotomy-antrectomy combination, had an ulcer recurrence rate of 5% or less and a very low post operative mortality rate. Unfortunately, postoperative symptoms including dumping syndromes continued to occur.

A proposed advance in ulcer surgery was selective vagotomy. Selective vagotomy denervated the stomach but spared the hepatic and celiac branches. A drainage procedure was still required. It was hoped that this procedure would result in fewer postoperative digestive sequelae. Unfortunately, trials could demonstrate no advantage of this procedure over traditional anti-ulcer surgery[10]. The progression of physiologic surgery for peptic ulcer disease continued in 1970, when Johnston *et al*[11] in England as well as Amdrup *et al*[12] in Denmark separately successfully performed parietal cell vagotomy also known as highly selective vagotomy. Highly selective or parietal cell vagotomy denervates the parietal cell mass but preserves innervation of the distal stomach (the motor unit). There was a flurry of enthusiasm for this operation. However, the procedure required operative finesse and training which was best acquired in an operating room working with a surgeon who has demonstrated good results with PCV[13].

Great advances in medical management of peptic ulcer disease began in 1977 when histamine H_2 antagonists were introduced to the market. Further improvement in medical treatment of peptic ulcer disease came in 1988 when a proton-pump inhibitor, omeprazole, was introduced to the market. Advances in medical therapy would go on to almost eliminate "failure of medical management" in peptic ulcer disease. Before the advent of H_2 receptor antagonists 50 percent of all duodenal ulcer operations were performed for intractable pain. Today most symptoms are adequately managed by medications. In fact, some believe that intractability may be due to noncompliance with and not resistance to medical therapy[14]. Another dramatic discovery occurred in 1982 (work published in 1984) when the association between *H pylori* infections and peptic ulcer was identified[15,16]. It was astonishing to believe that in the majority of patients, peptic ulcer was an infectious disease. In 1994, at the Consensus Development Conference, the National Institutes of Health panel concluded that infection with *H pylori* plays a significant role in the development of ulcers and that optimal treatment involves combination therapy using histamine H_2 antagonists or proton-pump inhibitors coupled with broad-spectrum antibiotic therapy. The medical literature of the world is replete with studies regarding *H pylori*; and there is now clear evidence, and general acceptance, that treatment with eradication of *H pylori* can cure peptic ulcer.

Today medical therapy cures peptic ulcer in the vast majority of cases. Therefore in many areas of the world elective surgery for peptic ulcer disease has all but disappeared[17]. Surgical management of peptic ulcer disease is still useful in cases of drug failure or for patients unable to obtain the drugs or to comply with medical therapy. In most parts of the world, surgical therapy is now utilized primarily for complications of peptic ulcer.

These are usually emergency operations. Currently up to 90 percent of all ulcer operations are interventions for complications including hemorrhage, perforation, or obstruction[14]. While the numbers of elective ulcer operations have plummeted there is conflicting data on whether the incidence of complications has remained unchanged, decreased, or even increased over the years. Some believe that the need for emergency surgery has not fallen, probably because of the increasing incidence of NSAID-associated complications[17]. A recent review from California indicates that acute surgical emergencies for bleeding or perforated ulcers are on the rise and mortality remains high[14]. Others feel that proton-pump inhibitors and *H pylori* eradication have resulted in fewer emergency operations for peptic ulcer disease[18]. Recent data from Finland from 1987-1999 revealed that elective surgery for peptic ulcer disease has virtually disappeared and that surgical mortality from ulcer surgery has remained unchanged (8%) over the last 15 years[19]. The stable mortality rate probably reflects a balance between an improvement in overall care but in an aging population.

Current surgical options for the management of peptic ulcers and their complications include truncal vagotomy and pyloroplasty, truncal vagotomy and antrectomy, parietal cell vagotomy (PCV), subtotal gastrectomy, patch repair for ulcer perforation, and oversewing of bleeding vessels. Older literature recommended that the choice of operation be based on factors such as patient age, comorbidity and nutritional status[20]. Since 1990, the trend has been towards more minimal operations. This is due to the belief that most patients are either on NSAIDS or will be found to be *H pylori* positive.

Several series have suggested patients with complicated peptic ulcer (bleeding or perforation) are less likely to be *H pylori* positive compared to non-complicated ulcers[21-23]. Others refute this, finding high *H pylori* infection rates in patients with perforated ulcers[24,25]. With bleeding ulcers; a major concern is false negative *H pylori* tests[26]. Thus the rapid urease test is unreliable in the face of bleeding[27]. and even results with the polymerase chain reaction for *H pylori* are controversial[28,29]. Although the role of *H pylori* infection in complicated ulcer disease, however, remains controversial, several clinical trials demonstrate that patients with eradication of *H pylori* infection have a reduction in rebleeding rates and rate of ulcer recurrence[30-33]. Similarly, the rate of recurrent ulcer disease following perforation also has a demonstrable association with persistent *H pylori* infection, and post-operative eradication seems to be a necessary step in the management of these patients[24,25,34]. Further, patients with gastric outlet obstruction have been shown to have better outcomes, with fewer recurrences or complications when *H pylori* eradication is a part of their therapeutic management[35].

With the current understanding of pathophysiology most surgeons feel that it is prudent to pursue a conservative approach in the majority of patients. This includes repair of perforations or oversewing of bleeding vessels without performing a definitive ulcer operation. This approach is supported by modern medical regimens designed to eradicate *H pylori* infection[3]or by the cessation of NSAIDS[37]. It has been demonstrated that *H pylori* eradication may significantly reduce the rate of duodenal ulcer re-bleeding, and that if the infection is cured, the preventive effect may be maintained for at least 48 mo[31].

Recent data available suggests that perforations caused by *H pylori* and NSAIDS may be two distinct entities, and supports a policy of patch repair followed by eradication of *H pylori* in those who are infected[38]. A series of more than one hundred patients with perforated duodenal ulcer treated by simple closure of the perforation and *H pylori* eradication therapy versus simple closure and omeprazole revealed far lower ulcer recurrence rates in the *H pylori* eradication group[34]. Several other series support this approach[39,40]. In addition, a survey of surgeons in the United Kingdom reveals a conservative bent towards ulcer surgery in that nation[41]. However, it is essential to recognize that the recommended approach of patch repair and antibiotics for *H pylori* for peptic ulcer perforation treatment may never be supported by trial data comparing minimal surgery and *H pylori* eradication to an anti-ulcer operation[42]. Reasonable exceptions to the conservative approach in treating complications of peptic ulcer disease may be seen in patients who have failed an anti- *H pylori* regimen, have suffered other ulcer complications, have dependency on chronic NSAID therapy, or are known to be *H pylori* negative[36]. Also, the role (if any) of *H pylori* in ulcer recurrence after peptic ulcer surgery is unclear[43-47]. Virtually all anti-ulcer operations have now been performed laparoscopically. Laparoscopic patch repair of perforated duodenal ulcers has been widely applied with success[48]. Laparoscopic parietal cell vagotomy has been performed using a variety of techniques[49]. Widespread use of this operation is again unlikely for the same reasons that open PCV has dropped from favor. With improvements in instrumentation and robotics, minimally invasive gastric resections will become less formidable. Certainly truncal vagotomy via laparoscope is not difficult for any surgeon versed in laparoscopic anti-reflux surgery. However, while these approaches become readily available, their need is diminishing.

Future trends may be impacted by swings in the HP pendulum. As mentioned, some suggest that the incidences of HP infection in complicated ulcers might be lower than in non complicated ulcers[21-23]. Three recent series suggest that the HP infection rate in complicated ulcers is between 81% and 93%[24,25,34]. In contrast some feel that the rate of HP infection in ulcer patients is decreasing[50]. Widespread eradication of *H pylori*, may result in the emergence of idiopathic (no-*H pylori* and no-NSAIDs) ulcers. Such a trend may reverse the current conservative approach to the surgical treatment of ulcer complications. Finally, one must consider the impact of all trends on surgical training. An examination of trends in American surgical residencies is illustrative. For all gastric-related surgery, the average reported cases per chief resident ranged from 9.8-12.4 with a peak in 1990 and a nadir in 1999; in 2001 the reported case average was 11.3. Over the same interval, vagotomy decreased from 24% in 1990 to 7% in 2001[51]. Graduating chief residents in surgery in the United States have performed (on average) less than 1 parietal cell vagotomy (PCV) during their residency. The

average number of all types of vagotomy for graduating chief residents was 3.5, including 3.2 truncal vagotomies and 0.3 PCV procedures. Because residents may never see a PCV performed during their residency, it is possible that PCV, once in the mainstream of surgery, is obsolete[52].

In conclusion, the understanding of the pathophysiology of peptic ulcer and the development of powerful pharmaceuticals has had a great impact on the practice of surgery in this disorder. The discovery of and treatment for *H pylori* in peptic ulcer disease has affected surgical approaches. Complications of peptic ulcer disease have not gone away although they are being treated non-surgically with increasing frequency. When surgery is required for complicated peptic ulcer disease most surgeons now favor a minimalist approach with closure of perforations or simple over sewing bleeding vessels but without a definitive anti-ulcer operation. When the next generation of surgical residents finish their training will ulcer surgery even exist, or will it simply be a historical topic in surgical textbooks? Will case numbers drop to the point where we won't have surgeons who are trained in performing these operations or will endo-luminal approaches eliminate the need for these operations? Surgery for unremitting hemorrhage from gastritis has disappeared. Considering the advances made in the twentieth century is there reason not to think that with future advances anti-ulcer surgery will go the same route?

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TOPIC HIGHLIGHT

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Evolution in the treatment of metastatic colorectal carcinoma of the liver

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Abstract

Metastatic colorectal cancer to the liver is associated with a uniform poor prognosis without treatment. Advances in therapy over the past decades have now allowed surgical resections of the liver to occur with a low morbidity and mortality. Improvements in chemotherapy regimes have paralleled technical improvements and now allow a new group of patients to become eligible for surgical resection. This chapter will review the recent advances in surgical and chemotherapeutic regimes in metastatic colorectal cancer to the liver.

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INTRODUCTION

Colorectal cancer is the 3rd leading cause of cancer related deaths in males and females. In 2005 it is estimated to afflict 72 915 males and 71 004 females in the United States. (www.cancer.org) Autopsy studies have shown that the liver is the most common site of distant disease in colorectal cancer (Welch J 1979), and approximately 60% of patients will develop liver metastases at some point during the course of their disease.(Kemeny 2005)

Malignant tumors of the liver were once considered a lethal disease. Untreated metastatic colorectal carcinoma has a median survival of 6 to 9 mo. (Khatri 2005) Today multiple studies have demonstrated the safety and efficacy of hepatic resection. This chapter will review the history and evolution of hepatic resection for metastatic colorectal cancer to the liver.

SURGICAL CONSIDERATIONS

While reports of liver resections extend back from before the 19th century (Keen 1899), there was significant mortality associated with these procedures. Couinaud, Goldsmith and Healey substantially aided our understanding of the segmental anatomy of the liver and this has contributed substantially to the development of safe hepatic surgery. (Healey 1953; Couinaud 1954; Goldsmith 1957; Couinaud 1999) Surgical resection is the primary treatment for metastatic liver lesions, as patients who do not undergo resection do not survive 5 years. (Scheele J 1995) Large studies by experienced hepatobiliary surgeons have demonstrated a mortality of less than 5% for liver resections (Coppa 1985; Fong 1999), with a five year survival of 20%-38% (Gayowski TJ 1994; Fong Y 1997) for lesions confined to the liver. Additional studies have demonstrated that larger tumors and multiple tumors can also be removed safely (Adson 1980; Adson 1984; Fong 1999). An area undergoing evolution is extrahepatic spread, which traditionally was considered a contraindication for surgery. However, colorectal metastases to the lung do warrant resection in patients along with liver metastases (Regnard JF 1998), and more recently, 5-year survival after surgery for hepatic and extrahepatic disease has been reported to be as high as 29% in carefully selected patients (Elias 2004). Future randomized trials will have to be performed to delineate if resection of extrahepatic disease constitutes a sustained survival benefit for patients.

Optimization of surgical techniques has also aided in reducing peri-operative mortality from liver resections. Measurements of functional liver reserve can be evaluated by bilirubin, prothrombin time, and percent indocyanine green retention. (Miyagawa S 1995) If a large resection is planned, or there is concern regarding the function of the remaining liver, pre-operative portal vein embolization (PVE) offers a method to maximize the size of the hepatic remnant. (Makuuchi M 1990) A study of patients with

metastatic colorectal cancer to the liver demonstrated the efficacy of PVE. Preoperative PVE was performed in patients with a CT-scan estimated post-resection volume of 40% or less, all of whom had received pre-operative chemotherapy. The median increase in CT scan estimated liver volume was 42%, and 63% of the patients were able to undergo an extended liver resection (17/19 resections involved 4 or more segments). (Azoulay D 2000)

INFUSION PUMPS

As systemic therapy for colorectal liver metastases was historically ineffective, studies were undertaken to look at direct infusion of higher doses of chemotherapy directly into the hepatic arterial system. Cancers of the liver have been shown to primarily derive their blood supply from the hepatic artery. (Breedis C 1954) Therefore, chemotherapy delivered through the hepatic artery will directly target the cancer cells before the drug has an opportunity to be metabolized. The rationale behind hepatic directed chemotherapy is based on three principles. (1) a five fold increase in the concentration of drug reaching the liver by avoidance of initial systemic dilution (20% of systemic blood flow goes to the liver); (2) the arterial supply to tumors and predominantly portal venous supply to normal liver increases drug delivery to the tumor with relative sparing of normal liver tissue; (3) hepatic metabolization of certain drugs reduces the hepatic venous concentration minimizing systemic toxicity. Confirmation of the benefit of hepatic administration *vs* portal administration of chemotherapy comes from a small randomized trial in which patients received chemotherapy with floxuridine via the hepatic artery or the portal vein. Fifty-percent of patients receiving arterial chemotherapy responded to treatment, but, there were no responders in patients receiving chemotherapy via the portal vein. One third of patients from the portal treatment group who crossed over to arterial chemotherapy subsequently had a response to treatment. (Daly JM 1987)

Systemic (IV) chemotherapy was first compared to hepatic artery infusion (HAI) chemotherapy in patients with unresectable colorectal liver metastases randomized to receive 5-fluorodeoxyuridine (FUDR) IV or by HAI. Fifty percent of patients receiving HAI infusion had a decrease in measured tumor burden, compared with a 20% response rate for systemic IV infusion ($P = 0.001$). Some patients with tumor progression on IV therapy crossed over to HAI therapy and 33% of these patients responded or had stabilization of disease. Patients receiving HAI had a higher incidence of extrahepatic disease compared to the systemic IV treatment (56% *vs* 37%, $P = 0.092$). Complications of HAI treatment included biliary sclerosis (8%) and ulcers (17%). (Kemeny N 1987) A similar randomized trial from the National Cancer Institute also demonstrated the efficacy of HAI chemotherapy. Sixty-two percent of patients receiving HAI had tumor regression, while only 17% of patients receiving IV therapy had a response. HAI patients had significant rates of biliary sclerosis (21%) and ulcer disease (17%), however, HAI therapy improved survival benefit at two-years to patients without hepatic artery nodal disease. (Chang AE 1987) Additional randomized trials have shown an improved tumor regression and preservation of quality of life with HAI infusion in carefully selected patients compared to selected controls. For example, the survival benefit existed only in the subset of HAI patients with less than 30% liver involvement, treated at specialized centers, and only when the comparison group received either systemic chemotherapy or only supportive care. (Rougier P 1992; Allen-Mersh TG 1995) A meta-analysis of all treatment groups at this time did not demonstrate a survival benefit for patients receiving HAI therapy over systemic therapy. (Harmantas A 1996) However, a more recent prospective trial has shown a survival benefit of HAI. In this trial 117 patients were randomized to IV versus systemic 5-FU and leucovorin. Similar to other trials, an increased response rate (47% *vs* 24%, $P = 0.12$), and time to hepatic progression (9.8 *vs* 7.3 mo, $P = 0.034$) was seen in HAI as compared to systemic therapy. Despite an increase in extrahepatic disease progression, overall survival was improved in patients with HAI (24.4 *vs* 20 mo, $P = 0.0034$). Importantly, quality of life assessments also showed an improvement in physical functioning at 3 and 6 mo of follow-up. (Kemeny N 2006)

The technique to place the catheter for drug delivery has been modified over the years such that current treatment involves an open laparotomy to cannulate of the gastroduodenal artery, and cholecystectomy. An analysis of 544 consecutive hepatic artery pumps in patients with unresectable colorectal cancer, revealed increased complications in patients with variant anatomy, pump insertion into an artery other than the GDA, and placement by a surgeon performing < 25 cases. However, the study also showed that only 16% of pumps had failed by two years. (Allen P 2005)

In 1995 Moertel demonstrated the advantage of adjuvant chemotherapy in Stage III colorectal cancer (Moertel C 1995). As hepatic resection became a safer procedure, investigators began to question whether resection followed by adjuvant therapy could offer a survival benefit as well as reduce liver recurrence. The German Cooperative study randomized patients to surgical resection alone versus surgical resection and HAI of 5-FU and folinic acid. Interim analyses did not demonstrate a conclusive survival benefit and the study was terminated. (Lorenz M 1999) However, an additional randomized trial demonstrated a survival benefit for HAI. Patients after liver resection received either HAI with floxuridine, dexamethasone, and, IV fluorouracil and leucovorin, or, systemic therapy alone of fluorouracil and leucovorin. Survival at two years was increased in the group receiving combined HAI and systemic therapy (86 *vs* 72%, $P = 0.03$).(Kemeny N 1999) An intergroup study randomized patients to surgery alone *vs* adjuvant HAI FUDR with IV 5-FU. While 4-year recurrence rate and liver recurrence-free rate were improved with therapy (25% *vs* 46%, $P = 0.04$, and 43% *vs* 67%, $P = 0.03$), the improvement in overall survival did not reach stastical significance. (Kemeny MM 2002) This may have been secondary to the fact that a significant number of patients that were disqualified from the study at the time of surgery, or, that the performance of the study at multiple institutions introduced another

variable.

In summary, HAI can be performed safely in institutions with appropriate experience. In patients with resectable tumors, HAI may offer a survival benefit either alone or when used in conjunction with systemic therapy. The patients with advanced disease that will receive the most benefit from HAI are those without peri-portal nodal disease.

CHEMOTHERAPY

For many years the only chemotherapy option in advanced colorectal cancer was the thymidylate synthase inhibitor 5-FU, given in combination with folinic acid (leucovorin), to potentiate the effect of 5-FU. These drugs had been shown to prolong disease free survival, with a large meta-analysis demonstrating a response rate of 32% and a median survival of 11.5 mo. (Project 1992) The development of newer agents has significantly influenced the management of advanced colorectal cancer.

Oxaliplatin is a third generation platinum drug that forms DNA adducts and results in cell death, with more efficacy and less side effects than previous generation platinums such as cisplatin.(Woynarowski JM 1998) Oxaliplatin used in combination with 5-FU and leucovorin improves disease free survival when used as an adjuvant in stage II or III colorectal cancer.(Andre 2004) In advanced colorectal cancer, the use of oxaliplatin with 5-FU/LV (FOLFOX) improved response rates to 50.7% and increased median survival, although not significant, to 16.2 mo.(de Gramont A 2000)

CPT-11(Irinotecan) and its active metabolite SN38 inhibit topoisomerase I, resulting in single strand DNA breaks and inhibition of DNA synthesis. (Kawato Y 1991) A randomized trial of irinotecan and 5-FU/Leucovorin (FOLFIRI) demonstrated an improved response rate of 39% and improved median survival of 14.8 mo in patients with metastatic colorectal cancer ($P = 0.04$ versus 5-FU/LV). (Salz L 2000) More recent trials have looked at treating patients with FOLFIRI or FOLFOX and with disease progression, transferring the patient from irinotecan to oxaliplatin or vice versa. This trial demonstrated impressive improvement in survival of 21.5 and 20.6 mo, limited only by toxicity profiles.(Tournigand C 2004)

As research in cancer therapy has progressed, more specific targets of cancer cells have been identified. Cancer cells express vascular endothelial growth factor (VEGF) in an effort to promote new vessel growth and tumor survival. Bevacizumab is humanized monoclonal antibody against VEGF. A randomized phase 3 trial of bevacizumab in combination with irinotecan, fluorouracil and leucovorin (IFL) versus IFL alone demonstrated increased disease free progression (10.6 *vs* 6.2 mo respectively) and increased survival (20.3 mo *vs* 15.6 mo). (Hurwitz H 2004) The epidermal growth factor receptor (EGFR) is an integral part of the cell that is the target of many signals regulation cell growth and death. A monoclonal antibody against EGFR, cetuximab, has also been demonstrated to prolong survival in patients with colorectal cancer refractory to irinotecan (8.6 *vs* 6.9 mo). (Cunningham D 2004)

NEOADJUVANT CHEMOTHERAPY

The armamentarium for treatment of metastatic colorectal cancer has improved over the years. While surgical resection offers the best outcome, many patients are not resectable and therefore undergo either HAI or systemic chemotherapy as described above. Bismuth was the first to demonstrate that preoperative chemotherapy could downstage tumors and make surgical resection an option. Patients in this study ($n = 330$) had nonresectable colorectal liver metastases, based upon tumor size, location or number of metastases, and underwent treatment with 5-FU, leucovorin, and oxaliplatin. Fifty-three patients responded to chemotherapy such that they became eligible for hepatic resection. The overall 5-year survival of patients undergoing surgery was 40%, with no peri-operative mortality. While 64% of patients had a hepatic recurrence, almost one-half were able to undergo an additional hepatic resection. (Bismuth 1996) These results have been substantiated in additional trials using the same chemotherapy with five-year survival of 35%-50%.(Giacchetti 1999; Adam R 2001)

Analysis of unresectable patients undergoing HAI infusion has only identified a small number of patients that became amenable to surgical resection. The first study was a retrospective analysis of 5-FU based HAI in patients with colorectal and non-colorectal liver cancer. Surgical resection became an option in 5.8% (14/239) of patients. Of these patients, 9 had metastatic colorectal cancer and underwent surgery. Five patients remained free of disease at a mean follow-up of 36 mo. (Elias D 1995) A similar retrospective study by Meric et al identified 383 patients with metastatic colorectal cancer who received HAI with FUDR. Twenty two patients had a response such that they were able to undergo additional procedures. Ten were able to undergo a resection alone while 6 underwent resection with cryotherapy or radiofrequency ablation. At a median post-operative follow up of 17 mo, the majority had recurrence and only 1 patient was alive without evidence of disease. (Meric f 2000) A phase I trial of the chemotherapy regimen of HAI of FUDR and dexamethasone with systemic oxaliplatin ± irinotecan or 5-FU/LV, demonstrated safety and an improved response rate when compared to systemic chemotherapy alone. Despite the fact that the majority of these patients had progression of disease on previous chemotherapy regimens, and 87%-90% had a partial response, seven of thirty-six patients were able to undergo liver resection and 2 had a complete pathologic response. (Kemeny N 2005) These encouraging results warrant further studies on the role of HAI with systemic chemotherapy in patients that fail to respond to other first line chemotherapy regimens.

The ability to respond to neoadjuvant chemotherapy improves survival and may be a significant harbinger of long-term survival. Allen studied patients presenting with colorectal liver metastases presenting within 1 mo of the primary surgery. All patients were resectable by radiological criteria. One group of patients went directly to surgery while the other group of patients received neoadjuvant chemotherapy consisting of 5-FU in combination with either oxaliplatin or irinotecan. The administration of

chemotherapy did not statistically influence survival, and no patients became radiographically unresectable while on chemotherapy. However, the patients on chemotherapy that had stabilization or regression of disease, had significantly improved survival over those patients that had disease progression on chemotherapy. (87% versus 38% 5-year disease specific survival, respectively, P = 0.03). (Allen P 2003) While this paper demonstrates the predictive value of response to chemotherapy, it also shows that patients who had disease progression also benefited from surgical resection.

CHEMOTHERAPY AND HEPATIC TOXICITY

With the knowledge that pre-operative chemotherapy can be given to downstage patients and possibly select those patients that are going have an improved survival, one must consider the effect of the chemotherapy on the liver. A retrospective analysis of 67 patients after liver resection revealed significant increase in morbidity in the group that received pre-operative chemotherapy consisting of 5-FU combined with either oxaliplatin or irinotecan. While there was no mortality, post-operative complications occurred in 37.8% of patients receiving neoadjuvant therapy and only in 13.6% of patients undergoing resection alone (P = 0.03). Transient hepatic failure occurred in 5 patients in the chemotherapy group. Pathologic examination of liver outside of the tumor revealed significantly increased sinusoidal dilatation in the chemotherapy group (49% *vs* 25% respectively, P = 0.005). There was no difference in steatosis or fibrosis. Multivariate analysis revealed preoperative chemotherapy to be one of the factors associated with increased post-operative morbidity. (Karoui M 2006)

Nodular regenerative hyperplasia (NRH) is the result of hyperplasia of hepatocytes with obliteration of surrounding liver in the absence of fibrosis. (Steiner 1959) The result is an enlarged liver with large nodules and at times, portal hypertension that mimics cirrhosis. The inciting factor of NRH is not known. A large study of 2500 autopsies demonstrated an incidence of only 2.6%. In this study, there was an increased incidence in older patients with collagen vascular diseases, tumors, and with underlying cirrhosis. (Wanless 1990) Pathologic analysis often noted thrombosis or diminutive portal veins, suggesting that alterations in portal flow influenced the development of NRH. Additional support for modifications in blood flow comes from two described cases of congenital absence of the portal vein, where a liver biopsy confirmed NRH, (Grazioli L 2000), however, many additional studies of NRH have not demonstrated vascular abnormalities.

NRH has also been shown to occur after chemotherapy. A consecutive study of hepatectomy specimens identified NRH in 7% of specimens. (Washington K 1993) All these patients had received pre-operative 5-FU regimen for colon cancer metastatic to the liver. However, there remains a disparity between drug administration and development of NRH, as not everyone in this study who received chemotherapy developed NRH. More recent studies have begun to describe cellular mechanisms of NRH. Notch signaling is a highly conserved intracellular signaling pathway that is fundamental in cell differentiation and embryonic development. In mice and humans, there have been four NOTCH receptors described (NOTCH1-4). In mice, loss of notch signaling is a fatal event. However, if one of the receptors of Notch signaling (Notch1) is inactivated early in postnatal development, this leads to NRH in the absence of vascular abnormalities.(Croquelois 2005) In addition, overexpression of IL-6 has also been shown to lead to NRH in mice.(Maione D 1998) These studies demonstrate pathways that could be targets for future understanding and prevention of NRH.

A retrospective study analyzed adjacent liver in resected liver specimens for metastatic colorectal cancer. All patients received 5-FU, leucovorin and either oxaliplatin or irinotecan. NRH was demonstrated in 4% of specimens. Importantly, 51% of specimens had sinusoidal dilatation with one-half having additional venoocclusive fibrosis. This was most common in patients that had received oxaliplatin therapy. (Rubbia-Brandt 2003)

Bevacizumab has anti-angiogenic properties and it is not yet known if livers resected after this therapy will have alterations in post-resection regeneration or function. At Yale, we have performed hepatic resections after 5-FU based therapy with oxaliplatin or irinotecan. In addition, some patients have also received bevacizumab. NRH has been frequently observed in patients receiving oxaliplatin particularly with extensive treatment. To date however this has not impacted negatively on the postoperative course or on hepatic regeneration.

FUTURE DIRECTIONS

With the progression in the understanding of colorectal disease new therapies will evolve. Instead of a surgical intervention to install an infusion pump to direct therapy into the liver, perhaps a therapy will be targeted to a gene. For example, adenoviral vectors are taken up by the liver and processed safely without evidence of inducing tumors (Bell 2005). These vectors can be used to carry cytokines, genes and apoptotic inducing proteins. Microarray technology has identified profiles of colorectal cancers that are likely to metastasize (Croner R 2005) and may help identify patients that need systemic chemotherapy. Gene profiling has identified characteristics that portend a favorable response to chemotherapy. (Matsuyama R 2006) The challenge will be to translate these studies into meaningful data for the patients with colorectal cancer.

In conclusion, the treatment of liver tumors has evolved substantially over the last century. The best chance for survival is surgical resection, and today liver resection can be performed with a low morbidity in correctly selected patients. Improvements in chemotherapy have improved survival in primary and metastatic colorectal cancer. Furthermore, chemotherapy has been able to downstage patients with advanced disease rendering them surgically resectable. Future studies will need to evaluate the hepatic side effects of newer drug regimens.

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TOPIC HIGHLIGHT

John Geibel, MD, Dsc and Walter Longo, MD, *Series Editors*

Ethical tensions in solid organ transplantation: The price of success

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Abstract

Solid organ transplantation has rapidly developed into the therapy a choice for end-stage organ failure. The expansion of its use has resulted is a large deficiency in organ supply. To address this, the field of organ transplantation has attempted to develop new strategies that would increase the availability of organs for transplant. Some of these strategies include expansion of the donor pool by increasing the number of living donors or using deceased donor organs that may be marginal or "expanded". The intent is to bring life-saving therapy to individuals in need; however, much of this expansion has been brought forward without clear prospective guidelines. This article focuses on the current disparity between organ supply and demand, and how this has impacted the use of living donors and development of the "expanded donor" concept.

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Key words: Ethical tensions; Organ transplantation; Donor

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INTRODUCTION

The field of solid organ transplantation has made dramatic improvements to the overall health of patients suffering from end-stage organ failure. The ability to provide successful organ replacement is a testament to the progress made in the development of modern immunosuppression and the refinements made in the surgical management of these patients. The subsequent expansion of solid organ transplantation is depicted in Figure 1 which demonstrates that currently more than 90 000 patients are awaiting some form of solid organ transplant in the United States. Unfortunately, this rapid success in organ transplantation has not been matched by a parallel increase in the supply of organs available for transplant. Figure 2 demonstrates this graphically, where the additions to the waiting list continue to increase in a nonlinear fashion while the number of recipients receiving organ transplants has increased little in the last five years. The disparity between individuals awaiting organ transplantation and the supply of transplantable organs is the single largest impediment to application of successful organ transplantation for

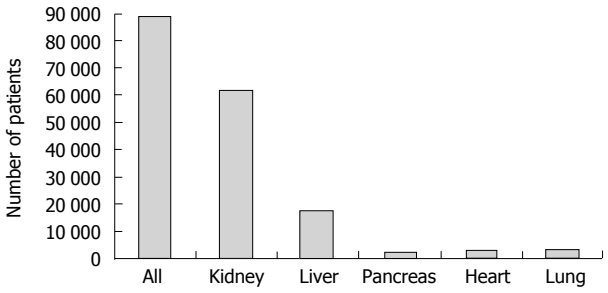


Figure 1 Number of patients on waiting list. May 12 2005 (www.optn.org)

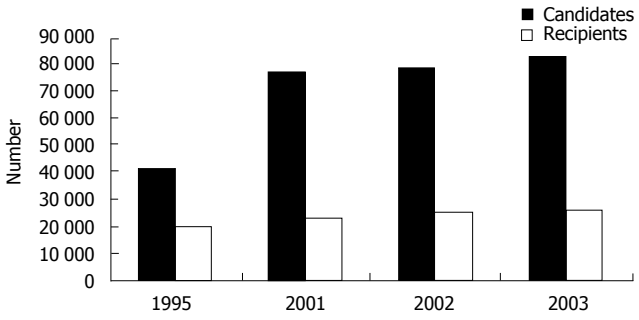


Figure 2 Growth of the number of candidates for transplantation compared to the number of recipients. (www.optn.org)

patients suffering end-stage organ failure.

One of the major ways transplant centers have attempted to increase the number of transplants performed is through increasing the numbers of living donors. Analysis of the types of organs used in organ transplantation shows that the year 2001 marks a significant shift to living donors. Figure 3 demonstrates in the year 2001, living donors were used more often as a source for kidney transplantation than were deceased donors. Analysis within the deceased donor pool reveals that as the median age of the donors has increased and as the severity of illness amongst the donors has increased, the use of marginal donors has played a greater role in supplying organs for transplantation. As the disparity between need and supply continues to widen, there are increasing pressures to seek alternative methods to supply organ donors to meet this crisis. An analysis of this disparity can most readily be divided between those issues that impact recipients directly and those that impact the number, type and quality of donors. With respect to recipients, the disparity between those listed and those transplanted can be regulated by imposing restrictions on the indications for transplantation. This, however, would be socially unacceptable as it is essentially withholding life-saving therapy. Another avenue to narrow the gap between those waiting for transplant and the supply of transplantable organs would be a change in the allocation policy. While allocation strategies continue to be modified within the organ groups (kidney, liver, pancreas, lung, heart) the intent of the new allocation strategies is to supply organs to those patients in the greatest need. The unwillingness to restrict the indications and the allocation of organs, demonstrates that society and the medial community will continue to broaden the inclusion criteria for patients who will benefit from transplantation. Surgical and immunosuppressive advances have markedly improved survival of all types of grafts of the past decade. From 1993 to 2002, the one-year graft survival of deceased donor kidneys improved from 82.1% to 89%, while live donor kidney grafts improved from a 91.4% to 95% 1-yr graft survival[1]. During this same time frame, pancreas grafts demonstrated improvement from 78.0% to 86.2%, while deceased donor liver grafts showed increases in graft survival from 73.9% to 82.4%. This continued growth in including more recipients is no doubt fueled by the success of transplant, both in quality of life and duration of life obtained.

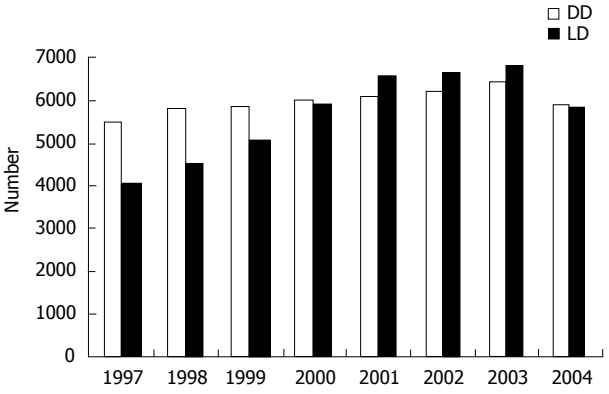


Figure 3 Comparisons of the number of transplants from deceased donors (DD) and living donors (LD). (www.optn.org)

EMERGENCE OF THE EXPANDED DONOR

Within the deceased donor category there are two broad distinctions of donor types. Those donors being designated as brain dead donors and donors designated as donation after cardiac death. The criteria for the diagnosis for brain death was established in 1968 by a NIH sponsored gathering of professionals. Documents coming out of this meeting, designated as the Harvard Brain Death Criteria[2], were instrumental in establishing brain death as medical and legal criteria to allow withdrawal of life support. At the time of this conference, there were many individuals being sustained on life support in intensive care units throughout the United States with no hope of recovery. This allocation of scarce ICU beds was negatively impacting the delivery of health care throughout the country. The establishment and acceptance of brain death criteria provided a legal, ethical and medical basis for the withdrawal of life support from these non-salvageable patients. It is from this pool that the majority of organ donors within the United States have been supplied. Although brain death criteria is accepted in North America, within other hemispheres, (i.e. Asia), brain death criteria has not been accepted due to religious and social beliefs. The efforts in the United States to increase the numbers of deceased donors have been varied and include public service announcements, education and legal mandates administered through regulatory agencies of health care within the United States. Despite these efforts, the conversion rate of deceased donors is below predicted values. However, even if all potential deceased donors were converted for donation, their numbers would be inadequate to supply enough organs for the increasing number of patients awaiting transplant[3]. Consequently, although a tremendous amount of resources have been applied to increasing the conversion rate within potential brain dead donors, the deceased donor pool would fall short of satisfying the disparity between those in need and awaiting solid organ transplantation. As a consequence, increasing the numbers of deceased donors have resulted in expansion of donation after cardiac death. Prior to the establishment of brain death criteria, the only deceased donors used for transplantation came from donation after cardiac death category. Though not formally recognized as such, donors after cardiac death should be considered to be a member of the expanded donor pool. Expanded donors are defined as organs that carry additional risks for transplantation beyond those associated with the standard transplant procedure[4]. This group of patients is typically composed of critically ill patients with no expectation of neurological recovery, in whom the decision to withdraw life support has been made. Within this group, various protocols have been applied to establish usability of the potential grafts[5]. Many organ procurement organizations throughout the United States have undertaken initiatives to increase the number of organs retrieved from this class of donor. Although acceptable within the defined limits for

kidney transplantation[6], this category of donor represents significant risks when applied to liver transplantation, specifically that of biliary complications and hepatic artery stenosis[7]. Despite the medical, legal and ethical justification for this procedure it is still shrouded in an era of suspicion by many members of the lay public and medical community. The suspicions rest with the perception that patients are prematurely withdrawn from life support to supply organs for transplantation. While the use of deceased donors from brain dead patients is considered standard of care within the transplant community, utilization of expanded donors has been evolving.

Within the specialty of kidney transplantation, a formalized definition of the expanded donor has been proposed (UNOS policy 3.5.1)[8]. This category of brain dead donor may carry the potential of increased risk of graft dysfunction and shortened graft survival; however, use of these kidneys would augment the current short supply of kidneys used for transplant and may appropriately serve the needs of recipients who otherwise would not be transplanted. Expanded donor kidneys, as defined by UNOS, are primarily all donors greater than 60 years of age or donors greater than 50 years of age who have co-morbidities of hypertension, cerebral vascular accident as a cause of death or creatinine elevation greater than 1.5. Institution of expanded donor utilization requires: definition of the expanded donor graft, identification of recipients who have given informed consent to be recipients of an expanded donor graft, and expedited placement of these kidney grafts to lessen the deleterious effects of cold storage time. Medical and ethical justifications for use of these expanded donors comes from analysis of SRTR (Scientific Registry of Transplant Recipients) data demonstrating that the relative mortality risk within the population of dialysis patients is lower with the use of an expanded donor kidney compared to remaining on dialysis at approximately 182 d after successful transplant and continues to have a survival advantage as long as the graft continues to work. What defines an expanded donor within the deceased donor liver grafts has been more difficult and currently there is no consensus. Within the deceased donor category, criteria such as age, infection, anatomic anomalies, presence of malignancies and steatosis carry with them added risk of liver transplants. Non-heart beating donor grafts derived from donation after cardiac death and livers that have been acquired through split procedures appear to have similar graft survival rates as standard deceased donor grafts, however, they are associated with more long-term complications[7,9]. Despite the obscurity of a true definition of expanded donor within the liver transplant community, many of these grafts are likely to expose the recipient to increased short term risk, including death, and may also result in more long term risks compared to standard liver grafts[10]. The balance, however, rests in the fact that increased waiting times are associated with increased mortality risks of those patients awaiting liver transplantation. It has been demonstrated by population analysis that the sickest patients awaiting liver transplantation do derive a survival benefit from transplantation with essentially any liver made available for transplantation[11]. Additionally, analysis of deceased donor liver grafts from a mid-west OPO demonstrates that there are few "perfect livers" available for transplant and in fact the number of livers included in the "perfect" category represent less than 3% of the livers used for transplant during the study (unpublished observations). Therefore, due to this spectrum of quality among the livers being utilized for liver transplantation, which extends from perfect livers to expanded donor livers, to livers that are not usable, the majority of deceased donor organs used for transplant are less than optimal.

It is not surprising that expansion of inclusion criteria within the deceased donor pool to include riskier organs for transplant has also stimulated expansion within the living donor category. 1954 marks a historic event in human history. It was at that time, December 24, 1954, when the first successful organ transplant was performed. This was a kidney transplant performed in Boston between identical twins. The necessity for using a living donor at that time was to overcome the immunologic barrier to transplant. Through the use of an identical, living donor the deficiency in immunosuppression therapy did not result in graft loss or patient loss. The ethical dilemma at that time was substantial and continues today. For the first time in medical history a healthy, volunteer donor was subjected to real and significant harm (surgical removal of a kidney) to provide benefit for another individual. Clearly this activity appears to violate the first rule of medicine *primum non nocere*. The justification for exposing the living donor to real and significant risks was balanced by the fact that the recipient who suffered end-stage kidney failure had no acceptable treatment options available. The diagnosis of end-stage kidney failure at that time resulted in the certain death of patients, as dialysis therapy was not available. Additionally, previous kidney transplant attempts resulted in graft loss secondary to rejection, or patient loss secondary to infection. This was, as Francis Moore described, "a desperate case". Additionally, legal intervention at the time justified exposure of the live donor as a trade-off in risks. Specifically, courts ruled that loss of an identical twin sibling would impose far more psychological harm and damage than would exposure to the risks associated with live donation. Since 1954, the inclusion criteria for acceptable, living donors has expanded. It has expanded with respect to organs being donated for transplantation as well as candidates being accepted for donation.

LIVING KIDNEY DONORS

Since 1954, the expansion of candidates deemed appropriate for living donation has moved beyond identical twins. With the development of effective immunosuppressive therapy, genetic identity is no longer required for successful transplant. This expansion began with genetically related donors, siblings or parents to donors that had an emotional relationship with the recipient including spouse and significant others. The increased use of living donors in kidney transplantation (Figure 3) has been further supported by the relative safety of the operation. Published reports demonstrate a

mortality risk of approximately 0.03% and a complication rate less than 3% when performed in high volume centers[12]. Additionally, surgical advances have improved the acceptance and willingness of living donation, as the movement from open nephrectomy to laparoscopic nephrectomy has demonstrated. Patients are more willing to undergo laparoscopic nephrectomy associated with earlier recovery and less pain associated with the donation. Analysis of population studies demonstrates that recipients of living donor grafts have decreased morbidity associated with transplant and an increase in patient and graft survival, as compared to deceased donor grafts. This improvement in survival of both patients and grafts is multifactorial and can be ascribed to earlier transplantation, limitation of cold ischemia time and transplantation with a better functioning graft. The most recent expansion of donor inclusion now includes directed, altruistic donation, non-directed anonymous donation and solicitation for donation. Directed, altruistic donation is usually provided by a donor who has a casual acquaintance relationship with the intended recipient. These donors usually originate from church groups, work organizations or other loose social associations. Because of the obvious benefits to the recipient and well defined and small risk to the donor, these donor-recipient pairs have been medically and ethically accepted by the transplant community. Further modifications of the living donor, recipient relationship include a swap donation between incompatible recipient donor pairs and other incompatible recipient donor pairs[13]. Previously, excluded groups have been the non-directed anonymous donors who now have become more acceptable within the transplant community. These donors are individuals who feel an overwhelming desire to donate a kidney to anyone on a transplant list for the sole purpose of improving their quality of life and longevity. This group was previously excluded on the basis of psychological instability; however, recent studies have demonstrated that although a significant portion of this group is excluded based on medical and psychosocial contraindications, there are individuals who are both psychiatrically and medically appropriate for donation[14]. Some programs have accepted these live donors for donation to patients on their transplant waiting list while others have accepted donation to the waiting list within the region. The most recent and unsettling practice within the living donor category has been outright solicitation for organ donation. This process most notably depicted through internet web sites appears to suffer from ethically questionable motives. In all forms of living donation the following practices exist. First, in directed donation, be it genetically related emotionally or casual acquaintance, there is a clear and definable benefit for which the donor can experience and value in the recipient. It appears that the risk benefit analysis under this relationship favors donation. In non-directed living donation, that being from a stranger or anonymous donor to either a transplant center waiting list or a regional waiting list, the benefit derived by the donor is in terms of a self-motivation and realization of doing a profoundly charitable and noble act for another human being. The benefit received by the recipient is obvious and the same for any recipient of living donor graft. Under this arrangement it appears that the risk benefit analysis would still favor the process of living donation. In both of these circumstances, the recipients participate in a well organized transparent and regulated system of valuation, regulation, allocation and access to the transplant graft. In the context of solicitation for donors, however, the emotional bond between donor and recipient pair is more difficult to define. The genetic bond is non-existent and motivations of pure altruism may take on a different a definition. Potential donors usually survey websites for the most compelling or set of characteristics that would satisfy their desire to donate. Reciepients have access in a non-equal way compared to other registered wait list recipients with UNOS. This injustice between access to secondary sources of organ allocation results in difficulties to delineate motives between donor and recipient pair, and provide the potential for exploitation of either or both participants in this enterprise, thus, making solicitation for living donation difficult to defend on ethical grounds. Donors are not prohibited from donating to the list in an anonymous fashion if altruism is their true motivation; however, recipients who undertake this process jump the queue among other recipients for access to this scarce resource. This unequal distribution of access undermines the trust and confidence of organ allocation within the US system.

LIVING LIVER DONORS

In the practice of liver transplantation, use of the living donor began as a case reports in the late 1989[15]. This was further studied in a prospective, comprehensive, IRB supported protocol at the University of Chicago[16], in which 20 donor-recipient pairs underwent transplantation. This study incorporated key components of the use of living donors, specifically; research ethics consultation, assignment of a donor advocate, demonstration of field strength of the institution and physician team prior to undertaking the study and a cooling off period with multiple steps in the informed consent process[17]. This landmark study in the use of living donors served to establish modifications in the living donor surgical procedure and facilitated incorporation and dissemination of the practice of living donor liver transplants for pediatric recipients world-wide. Justification for exposing donors to a potentially significant harm was offset by the dire circumstance of infants awaiting the liver transplant patient. Prior to institution of the living donor liver transplant procedure for infants, the mortality rates for infants awaiting liver transplantation approached 30%[18]. With the rapid acceptance of this surgical innovation, the mortality in some institutions performing pediatric liver transplantation diminished to less than 1%[19]. Throughout the study, the surgical techniques were modified and living donor liver transplantation for pediatric recipients has become standard of care worldwide. The estimated mortality risk for the donor is approximately 0.1%, with morbidity less than 15%[20]. It is important to note that within the use of living donors for pediatric liver transplantation, the majorities of donors are genetically or emotionally related to the recipient and include parents,

siblings, aunts, uncles and grandparents.

Extension of living donor liver transplantation to adult recipients began in the late 90's, from various institutions conducting independent small series[21,22]. Contrary to the pediatric experience, the introduction of adult-to-adult living donor liver transplantation did not benefit from a prospective, well defined, single center study. In fact, the majority of institutions performing adult-to-adult living donor liver transplant did so without an accepted IRB protocol. Consequently the risks and benefits associated with the procedure were difficult to quantify and not applicable across programs. Donor deaths have occurred and it wasn't until 2001 where the first widely publicized donor death stimulated regulatory intervention in New York State concerning adult to adult living donor liver transplantation[23,24]. Secondary to that well publicized complication, the numbers of adult-to-adult living donor liver transplants rapidly diminished within the United States, having only recently begun to increase[25]. Adult-to-adult living donor liver transplantation is performed in many countries throughout the world and in particular serves as the only source of organs for transplantation in regions where religious opposition to deceased donation occur (i.e. Japan and China)[26]. Unfortunately, the development of the procedure has not clearly delineated indications and contraindications for both recipients and donors. This is a process which continues in evolution and is dependent upon reports from individual centers. There is currently an NIH sponsored multi-institutional trial collecting data which should be very important to help define the appropriate role and complications of adult-to-adult living donor liver transplantation for both recipient and donors alike. Although the majority of donor-recipient pairs have both a genetic or emotional relationships justifying the risk/benefit of such a procedure, there has been increased inclusion of anonymous living donors and solicitation for living donors. In an operation whose mortality has been predicted between 0.6 and 1% and whose morbidity has ranged as high as 67% the donor population[27,28], it is difficult to ethically justify the undertaking of the riskiest live donor operation performed to date without understanding the benefit derived in the risk/benefit analysis. Living donation has also been applied to lung transplantation, although small in number it appears that well selected donors and recipient pairs result in an acceptable outcome justifying the exposure of the donor to this operation[29,30]. The relative safety to this operation is no doubt dependent upon the small number of centers who are engaged in this practice. With respect to living donor intestinal transplantation, it appears that in well controlled circumstances, when performed in experienced centers, this operation may be of benefit to recipients with acceptable risks to donors[31,32]. However, the number of cases remains small and the demonstration of an inadequate deceased donor supply have not fully justified exposure and utilization of the live donor for intestinal transplantation. Therefore, it is difficult at this time, outside of an experimental protocol, to ethically justify placing a donor at unknown risk for undergoing intestinal procurement for a unclear recipient benefit.

In conclusion, it is clear that as transplantation provides a successful treatment for a variety of diseases which result in end-organ failure and that the demand for organs will continue to out strip the supply. This disparity will continue to drive the practice of expanding the criteria for what is defined as an acceptable deceased donor or living donor. With respect to deceased donors, expansion of the inclusion criteria can only be accomplished at the margins currently defined as *expanded donor criteria*. Utilization of these grafts does result in added risks experienced by the recipient. It is imperative that these added risks associated with the use of expanded, deceased donor grafts are appropriately matched to the expected outcomes in the recipients for whom they are used. Specifically, it would not be ethically acceptable to use a 70 year old kidney in a 16 year old recipient unless there were significant and compelling reason to expose such a recipient to the risks and limited survival of such a graft. Additionally, it may not be acceptable to utilize a 21 year old perfect kidney in transplantation of a 75 year old recipient with multiple medical problems who has an expected life expectancy of less than 3 years. These are difficult decisions which have not been codified into practice plans currently. With respect living donor use for kidney transplantation, it appears that our current dilemma rests in the use of living donor grafts procured from solicited donors. The risk/benefit analysis has always favored performance of a relatively safe nephrectomy for the demonstrable benefits in the transplant recipient. This risk/benefit relationship has always been solidified and enriched by a donor-recipient relationship; be it genetic, emotional or acquaintance. In liver transplantation, it appears that until such time when reliable indicators exist to exclude expanded donor grafts, the survival advantage with transplantation continues to be a compelling argument in favor their use. In the field of living donor liver transplantation, adult-to-pediatric transplantation has been incorporated as standard of care in pediatric liver transplantation. Within the adult-to-adult living donor liver transplantation procedure, the extent of the donor operation, inclusion and exclusion of donor candidates, and inclusion and exclusion of recipients continue to require delineation. Under the current practice it appears that more formalized, investigation and study would be required to protect both recipient and donor pairs and to define, clearly, the indications and contraindications of adult-to-adult living donor liver transplantation. With respect to the use of all living donors, informed consent, donor follow up, and protection of donors, should be paramount in institutions undertaking this enterprise.

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REVIEW

Hepatic perfusion disorders: Etiopathogenesis and related diseases

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Abstract

In this article, we have reviewed the hepatic perfusion disorder (HPD), etiopathogenesis of HPD and corresponding diseases. Review of the literature was based on computer searches (PubMed, Index Medicus) and personal experiences. We considered HPD reflects perfusion differences due to redistribution of arterial blood flow among segments, subsegments and lobes of the liver. The plain CT scan findings of HPD manifests as triangular or wedge-shaped areas of low attenuation. On contrast-enhanced CT scan, HPD manifests multiple (or single) transient wedge-shaped, rotundloid or irregular appearance, homogeneous hyperattenuation (in less cases, hypoattenuation) during the hepatic arterial phase (HAP) and isoattenuated or slightly hyperattenuated areas during the portal arterial phase. Dynamic enhanced magnetic resonance (MR) features are similar to enhanced CT scan. Angiographic findings include non-opacification of portal vein on portograms or wedge-shaped segmental staining in arterial and parenchymal phases on hepatic angiograms. The causes of HPD are arterioportal shunts (APS), intrahepatic vascular compressions and portal vein occlusion, steal phenomenon by hypervascular tumors, vascular variations and any other unknown reasons. It is very important for radiologists to be familiar with the various appearances of HPD to avoid false-positive diagnosis of pseudolesions and not to overestimate the extent of the disease.

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Key words: Etiopathogenesis; Hepatic perfusion disorders; Diseases

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INTRODUCTION

Definition of HPD

Hepatic perfusion disorder (HPD) refers to perfusion differences among segments, subsegments and lobes of the liver caused by various etiologies. It is usually transient peripheral and triangular, wedge-shaped or rotundloid hepatic parenchymal enhancement during the hepatic arterial phase (HAP) and returns to normal attenuation/intensity on portal venous phase (PVP) on CT and MR dynamic enhancement, and segmental staining or segmental perfusion defect on angiograms. It usually has a straight margin, and contains normal vessels and without mass effect. With the improvement of temporal resolution of CT and MR, detection rate of this phenomenon has increased recently. However, the nomenclature of this phenomenon is different in different imaging modalities, HPD is a good nomenclature for this phenomenon suitable for any other imaging modalities. This article presents the nomenclature and history of hepatic perfusion disorders and their spectrum with etiopathogenesis.

Nomenclature and history

In 1981, Inamoto *et al*[1] and Nishikawa *et al*[2] first described segmental low attenuation areas within the liver not corresponding to mass lesions. This phenomenon was attributed to decreased portal flow. In 1982, Itai *et al*[3] reported transient hepatic attenuation difference (THAD) during the HAP on CT, and explained the pathophysiology and etiology of this phenomenon. In 1984, Matsui *et al*[4] described segmental staining on hepatic angiograms and portal vein occlusion on portograms (a sign of intrahepatic portal vein obstruction) in liver tumor cases. They also found that the areas of segmental staining on hepatic angiograms were the ones of hepatic parenchymal enhancement during the HAP on CT. In 1988, Itai *et al*[5] reported the findings of segmental intensity difference (SID) on MR images. They found that the SID was defined as a zone of homogeneously long T1 and long T2 signal intensity with segmental or subsegmental distribution not corresponding to the areas of the mass. In cases of SID, they studied MR images combined with other images, such as CT scans, ultrasound (US) and angiography, for corresponding abnormalities. The areas of SID were trapezoid hypoechoic areas contacting with the hepatic contour as disclosed on US studies. They also found that many cases with wedge-shaped defects showed by portography and segmental staining on arteriography revealed no areas of low attenuation on plain CT and MR images and no pathologic dif-

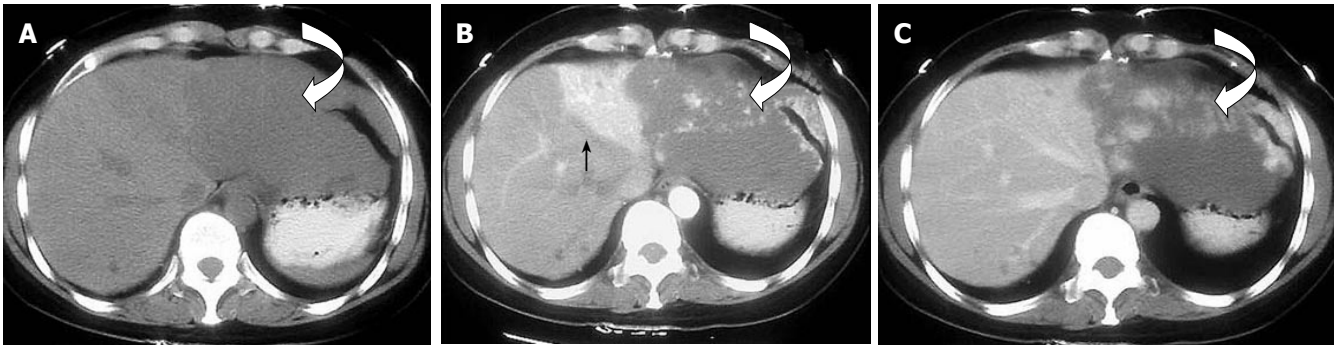


Figure 1 HPD in a 48-year-old woman with cavernous hemangioma. **A:** Pre- contrast administration transverse CT scan shows a large well-defined hypo-attenuating lesion located in segment Ⅱ (curved open arrow); **B:** HAP CT scan shows a nodular enhanced hemangioma (curved open arrow) and wedge-shaped segmental homogeneous hyperattenuating areas (solid arrow) adjacent to the tumor; **C:** PVP CT scan shows the still nodular enhanced hemangioma (curved) and the peripheral area resumed isoattenuating.

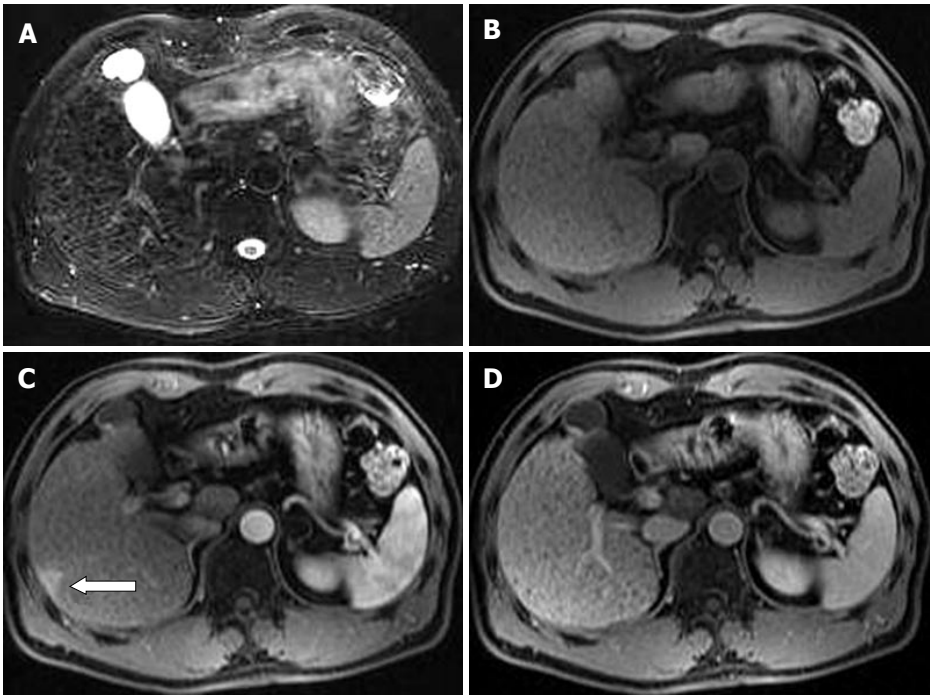


Figure 2 HPD in a 56-year-old man with liver cirrhosis. **A:** Fat-saturated breath-hold turbo FSE T2-weighted transverse MR scan shows multiple punctuate hypointensity cirrhotic nodules and surrounding reticulated fibrotic liver parenchyma, without any obvious hyperintense tumoral lesions; **B:** pre- contrast administration transverse spoiled gradient-echo T1-weighted MR image (118/4.1; flip angle, 80°) shows no obvious hypointense tumoral lesions observed; **C:** transverse contrast-enhanced T1-weighted MR image obtained during HAP shows a subcapsular small patchy hyperintense tumor-like lesion (pseudolesion) (open arrow); **D:** transverse contrast-enhanced T1-weighted MR image obtained during PVP shows no lesion in that area.

ferences at microscpic examination. In 1997, Gryspeerdt *et al*[6] reviewed the phenomenon of hepatic perfusion differences on dual-phase spiral CT scan, and first nominated it as hepatic perfusion disorders (HPDs). They considered that various HPDs are related to portal venous inflow obstruction, hepatic venous outflow obstruction (e.g., Budd-Chiari syndrome, cardiac failure, mediastinal fibrosis), mediastinal or thoracic venous inlet obstruction, focal liver lesions, inflammatory processes, normal anatomic variants in the hepatic blood supply, altered hemodynamics after the placement of a transjugular intrahepatic portosystemic shunt, and uncertain causes. In the same year, Lee *et al*[7] observed the correlation between the arterioportal shunt caused by liver biopsy injury and wedge-shaped early transient enhancement phenomenon of the liver during helical dynamic CT in 21 patients, and nominated it as transient subsegmental parenchymal enhancement (TSPE). After that, many authors reported this phenomenon as THAD and HPD[8-14]. However, THAD reflects an enhanced, redistributing arterial flow to a hepatic segment or lobe on CT; it cannot reflect other imaging modality findings, such as segmental staining on arteriogram, trapezoid hypoechoic areas on US and SID on MR. In our opinion, HPD is a good nomenclature for this phenomenon suitable for any other imaging modalities.

Imaging features

The plain CT scan findings of HPD manifests as triangular or wedge-shaped areas of low attenuation. The metabolic pathophysiology of the phenomenon is decreased glycogen and subsequent increase of fat and/or edema secondary to venous inflow obstruction due to portal branch occlusion. On contrast-enhanced CT scan, HPD manifested multiple (or single) transient triangular, wedge-shaped, rotundloid or irregular appearance, homogeneous hyperattenuation (in less cases, hypoattenuation when the hepatic artery is compressed or occluded) during the hepatic arterial phase (HAP) and isoattenuated or slightly hyperattenuated areas during the portal arterial phase (PVP) (Figures 1 and 2). Plain MR scan is defined as a segmental

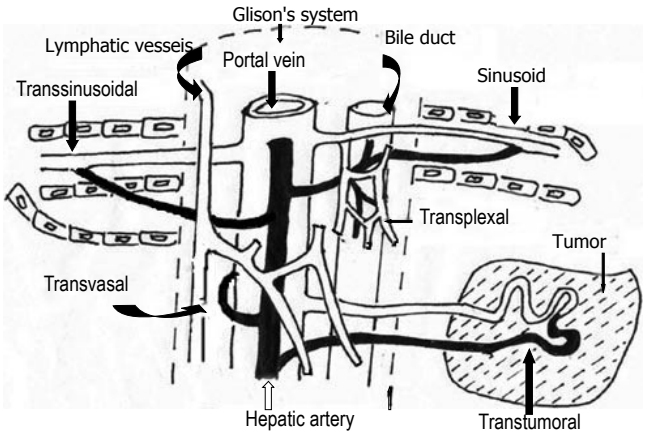


Figure 3 Schematic drawings showing hepatic microvasculature and arterioportal anastomoses or arterioportal shunts, including the transsinusoidal route (between the interlobular arterioles and portal venules or sinusoids) and the transplexal route (peribiliary plexus), transvasal route (perilymphatic vessels) and transtumoral route.

or subsegmental iso-intensity on T1- and T2-weighted images (in less cases, long T1 and long T2 signal intensity). Dynamic enhanced MR features are similar to enhanced CT scan. Angiographic findings included non-opacification of portal vein (a sign of intrahepatic portal vein obstruction) on portograms or wedge-shaped segmental staining in arterial and parenchymal phases on hepatic angiograms, and corresponding features are seen if there is an occlusion of hepatic vein or aberrant blood supply. There lacks special report of US features about HPD yet, trapezoid hypoechoic areas on US were found corresponding to SID on MR or THAD on CT images[6]. There is also no report about real-time contrast-enhanced US features of HPD as yet.

Table 1 The causes of HPD

The causes of HPD
Arterioportal shunts
Trauma
Neoplasms
Malignant tumors: HCC, cholangiocarcinoma, metastases
Benign tumors: hemangioma, FNH
Interventional procedures
Biopsy
Biliary and abscess drainage
Various physical treatment of hepatic tumor
Percutaneous transhepatic cholangioscopy
TIPS
Liver cirrhosis
Congenital APS
Intrahepatic vascular compression and occlusion
Neoplasms
Malignant tumors: HCC, cholangiocarcinoma, metastases
Benign tumors: hemangioma, FNH
Inflammatory changes
Hepatic abscess, acute cholecystitis, cholangitis.
Focal eosinophilic necrosis
Hepatic parenchymal compression
Rib compression
Perihepatic peritoneal tumors
Pseudomyxoma peritonei
Perihepatic fluid collections
Others
Right-sided heart failure
Pericardial disease
Budd-Chiari syndrome
Mediastinal fibrosis
Steal phenomenon by hypervascular tumors
HCC
Hypervascular metastases: islet cell tumors, carcinoids, renal cell carcinoma, and breast cancer
Vascular abnormalities and hereditary hemorrhagic telangiectasia
Vascular variabilities: capsular vein, accessory cystic vein, aberrant right gastric vein
Hereditary hemorrhagic telangiectasia
Unknown causes

PATHOGENESIS OF HPD

HPDs may be noted to accompany tumors; however, they may occur due to non-tumor vascular reasons as well. Among the reasons of HPD are arterioportal shunts, intrahepatic vascular compression and occlusion, steal phenomenon by hypervascular tumors, vascular abnormalities and hereditary hemorrhagic telangiectasia, and any other unknown reasons (Table 1).

Arterioportal shunts

A distinctive feature of the liver is its unique dual blood supply, which comes from the hepatic artery (25%) and the portal vein (75%). There can be several potential communications between the vessels, including transsinusoidal, transvasal and transplexal (peribiliary) routes (Figure 3). In some pathologic cases, an organic or functional communication between a hepatic arterial branch and the portal venous system occurs, resulting in redistribution of arterial flow into a focal region of portal venous flow due to high pressure in hepatic artery and low pressure in portal branch. On dynamic enhanced CT/MR scan or hepatic arteriography, HPD manifests as a transient area of high attenuation/intensity or segmental staining due to passage of contrast material from arterial blood into portal vein branch, thus enhancing/staining a focal area of the liver before the adjacent parenchyma is enhanced/stained through the portal venous system. The areas with APS are enhanced or stained intensively due to hyperperfusion; in contrast, the other areas without APS are enhanced or stained normally due to normal perfusion. The helical CT or MR findings of APS are as follows: (1) Early enhancement of the peripheral portal vein branches during the HAP and before the main portal vein is enhanced; and (2) enhancement of the peripheral portal vein branches and main portal vein without enhancement of the superior mesenteric and splenic veins. Hepatic arteriographic findings of APS are early visualization of small portal branches when hepatic artery is visualized.

The causes of APS include: (1) Trauma: Abdominal trauma sometimes causes an organic communication between the arterial and portal venous systems due to vascular rupture or produces functional APS due to portal or hepatic vein thrombosis; (2) Neoplasms: Hepatic neoplasms include both malignant tumors such as HCC, cholangiocarcinoma and various hypervascular metastases and benign tumors such as hemangioma and focal nodular hyperplasia (FNH)[14]. HCC is either the most common malignancy of the liver or the most common cause of HPD. In cases of peripheral small HCC, APS is induced

via the transtumoral route, and in cases of large HCC, APS is induced via the transvasal route (due to portal vein tumor thrombosis), the transtumoral route (through the tumor itself), or the transsinusoidal route (between microscopic hepatic arterioles and portal venules distal to portal vein compression or thrombosis) and a transplexal (peribiliary) route. Cholangiocarcinoma may cause APS when the portal or hepatic vein is compromised. Hypervascular metastasis may cause APS similar to HCC. It was considered that hepatic hemangioma seldom causes APS; however, recent studies have shown that there is transtumoral route of an arterioportal shunt between the hepatic artery and the portal vein[17-19]. The arterioportal shunt of a hemangioma is somewhat different from that of an HCC because most HCCs accompanying an arterioportal shunt tend to be large tumors with portal vein invasion. In small hepatic lesions < 3 cm, the incidence of arterioportal shunt is significantly higher in a small hemangioma than in a small HCC[20]. Small hemangioma with large arterial flow and flash filling has APS and shows HPD more frequently than a large one[17,18]. FNH is supplied by an anomalous enlarged hepatic artery and is neither supplied nor drained by the portal vein. We found that FNH showed HPD in two cases, although the pathogenesis is unknown. There was neither HPD observed in our practice nor articles reported in hepatic adenoma whether it may show HPD is unknown yet; (3) Interventional procedures: Interventional procedures (e.g., hepatic biopsy, biliary and abscess drainage, various physical treatment of hepatic tumor, percutaneous transhepatic cholangioscopy, and transjugular intrahepatic porto-systemic stent shunt) can produce iatrogenic arterioportal fistulas due to portal vein injury[21,22]; (4) Liver cirrhosis: Liver cirrhosis is caused mainly by parenchymal necrosis, which is followed by deposition of connective tissue, nodular hepatocyte regeneration, and distortion of the lobular and vascular hepatic architecture, and false lobules, producing portal hypertension. Liver cirrhosis is known to alter normal hepatic blood flow dynamics, resulting in increased arterial flow and decreased portal venous flow to the liver. APS is believed to be secondary to occlusion of the small hepatic venules and retrograde filling of the small portal vein branches by way of arterioportal anastomoses (transsinusoidal route)[23]. In addition, hypertrophy of the peribiliary plexus and direct anastomosis in the peripheral area of the liver are another routes of APS. Due to false lobules formation and distortion of the vascular hepatic architecture, HPD may appear as small enhancing nodules during HAP in cirrhotic patients and may be confused with small hepatocellular carcinomas. A correct diagnosis can be made by observing the portal phase (rapid wash out with hypoattenuating lesion in HCC, isoattenuating in case of HPD at CT) and the lack of T2 hyperintensity at MRI. MR with reticuloendothelial contrast agents (supermagnetic iron oxide, SPIO) may also be of help (signal loss in HCC, no signal loss in case of HPD); (5) Congenital APS: Some congenital hepatic diseases, such as infantile hepatic hemangioma, arteriovenous malformations (AVMs) and infradiaphragmatic total anomalous pulmonary venous return (TAPVR), can also be associated with APS.

Intrahepatic vascular compressions and occlusion

Intrahepatic vascular includes hepatic artery, portal and hepatic vein, and may be compressed and occluded by many causes: (1) Compressions and occlusion of hepatic artery: As a result of the compression and occlusion of hepatic artery, the hemodynamic balance between the portal vein and the hepatic artery in the affected lobe or segment may change. Different from common features of HPD, areas of hypoattenuation/intensity may be seen on contrast-enhanced CT or MR images and segmental staining defect may be seen on hepatic arteriograms due to decrease in the blood flow of the affected hepatic artery[16]; (2) Compression and obstruction of portal vein: When the compression and obstruction of portal vein occurs, perfusion alterations are produced by increases in arterial flow through transsinusoidal, transvasal, transtumoral, and especially transplexal (peribiliary) routes to compensate for the diminished portal venous flow (functional APS) and by decreased dilution of the contrast material by the non-enhanced portal venous flow[10,14,16]; (3) Compressions and obstruction of hepatic vein: Occlusion of the hepatic veins results in increased sinusoidal pressure and reverses the pressure gradient between the sinusoidal and portal veins. The portal vein then becomes a draining vein and there is an increase in hepatic arterial flow, resulting in a functional APS, as in liver cirrhosis[16].

The causes of compression and obstruction of intrahepatic vessels include: (1) Neoplasms: Hepatic tumors are either sometimes associated with portal vein compromise and or, less frequently, with hepatic vein and artery compromise. As a result of the invasion of the hepatic artery due to tumoral compression and encasement, the blood flow in the compromised areas decreases. The invasion of the portal and hepatic vein due to tumor may result in obstruction due to tumor thrombosis, blood flow decrease or stasis[10]; (2) Inflammatory changes: Local inflammation can cause hyperemia of the hepatic artery and stoppage of regional portal or hepatic venous flow, as in cases of hepatic abscess, acute cholecystitis, cholangitis. The compromised areas include both the inflamed lesion and the surrounding parenchyma[16]. Recently, there is a report that focal eosinophilic necrosis may cause HPD. Pathologically, focal eosinophilic necrosis is a focal area of hepatocellular necrosis caused by severe eosinophilic infiltration of perivascular space. Therefore, one may assume that eosinophilic infiltration of the perivascular space may be the cause of the stoppage of regional portal venous flow, portal thrombosis and occlusion[14]; (3) Hepatic parenchymal compression: When there is increased pressure on the hepatic parenchyma, blood flow through the portal vein is reduced due to portal venous low pressure system and consequently, increased arterial inflow to the affected hepatic segment due to the high pressure arterial system[11]. The causes of hepatic parenchymal compression include rib compression (in some cases, the medially concave rib may constantly compress the hepatic parenchyma), perihepatic peritoneal tumors, such as pseudomyxoma peritonei, and perihepatic fluid collections. This transient hemodynamic change is reversible when

the compression is removed; (4) Others: Other causes of compressions and obstruction of intrahepatic vessels include right-sided heart failure, pericardial diseases, Budd-Chiari syndrome, or mediastinal fibrosis, which may result in hepatic vein occlusion[10]. In patients with right-sided heart failure, the contrast agent can be seen within the hepatic veins during HAP due to decreased hepatic vein flow, while during PVP stagnation of the contrast agent within the sinusoids may give rise to a mottled appearance of the parenchyma.

Steal phenomenon by hypervascular tumors

Hypervascular tumors usually include HCC and hypervascular metastases, such as islet cell tumors, carcinoids, renal cell carcinoma, and breast cancer. These hypervascular tumors may result in "hypertrophy" of the hepatic arterial blood supply to the lobe or segment of the liver containing the tumor, and can "steal" arterial blood from the surrounding parenchyma due to siphoning effect, which is similar to the phenomenon of stealing blood caused by AVMs. Then the hepatic parenchyma adjacent to the tumor can receive more than usual arterial blood supply and the perfusion of the remainder of the lobe is reduced. The net effect of this stealing depends on the tumor location, degree of tumor vascularity, and ratio of tumor to parenchyma blood supply[10,25,26].

Vascular abnormalities and hereditary hemorrhagic telangiectasia

Vascular variabilities, such as capsular veins drain into adjacent hepatic sinusoids, accessory cystic veins drain into the liver parenchyma of segments IV-V, parabiliary system from the head of the pancreas, duodenum and distal stomach drain into the posterior part of segment IV, veins from the gastric antrum and pancreatic head into segments I and IV, which are third hepatic inflow tracts, may drain the systemic venous circulation into the liver[27-29]. Focal intensively enhanced areas are seen on HAP images of CT or MR, segmental staining is seen on arteriogram because of the early inflow of contrast material compared with the inflow into the surrounding parenchyma, which receives contrast material mainly from the splenic and superior mesenteric veins. Common locations of the HPD caused by vascular variations are adjacent to the gallbladder fossa, anterior to the porta hepatis, adjacent to the falciform ligament, and in the subcapsular area. These HPDs might appear either wedge-shaped and triangular; in less cases, they appear nodular, it makes difficult to differentiate from a true lesion, especially located in subcapsular areas[10]. Sometimes, hepatic arterial supply derived from the cystic artery (hepatic branch artery-ramus profundus) can also produce a hyper-perfusion in a pericystic location during HAP, similar to those produced by the cystic vein.

Hereditary hemorrhagic telangiectasia (HHT, Osler-Weber-Rendu disease) is a vascular disease with autosomal dominant transmission characterized by multiple telangiectases, which are thin-walled, dilated vascular channels with arteriovenous communications. HHT affects mucocutaneous tissue most frequently, but any part of the body can be affected, including the liver. The hepatic changes, including widened and tortuous hepatic arteries, telangiectases, arteriovenous and portovenous fistulas, connective tissue formation with fibrosis and atypical cirrhosis, high cardiac output, secondary congestive heart failure due to a left-to-right intrahepatic shunt, portal hypertension, gastrointestinal hemorrhage, ascites, or encephalopathy[30-32]. APSs are nearly universal in HHT, and HPDs often occur. Contrast-enhanced CT or MR shows heterogeneous perfusion of the hepatic parenchyma with multiple, peripheral, wedge-shaped or irregular areas of intensively enhanced areas during the HAP. Angiography shows diffuse mottled capillary blush with multiple segmental or subsegmental staining.

CONCLUSION

HPDs reflect a hemodynamic change in the normal dual blood supply of the liver in area of surrounding lesion. The nature of HPDs is perfusion differences due to redistribution of arterial blood flow among segments, subsegments and lobes of the liver. Among the reasons of HPD are arterioportal shunts, vascular compression and occlusion, steal phenomenon by hypervascular tumors, vascular variations and any other unknown reasons. It is very important for radiologists to be familiar with the various appearances of HPD to avoid false-positive diagnosis of pseudolesions and not to overestimate the extent of the disease. Because knowing the characteristics of HPDs and differentiating them may prevent the tumor diameter from being measured larger than they actually are in the case of hypervascular tumors such as HCC.

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BASIC RESEARCH

Effects of nuclei ambiguus and dorsal motor nuclei of vagus on gastric H^+ and HCO_3^- secretion in rats

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Abstract

AIM: To determine the effects of electrical stimulation of nucleus ambiguus (NA) and dorsal motor nuclei of vagus (DMV) on gastric acid and bicarbonate secretion in rats.

METHODS: NA and DMV in rats were electrically stimulated. Pylorus ligation or esophagus perfusion was used to collect the gastric secretion. The titratable H^+ quantum, H^+ concentration, HCO_3^- secretion quantum were measured.

RESULTS: Electrical stimulation of NA had no effects on the volume of gastric juice, titratable acidity and acid concentration, but elicited a pronounced increase in the total bicarbonate. However, electrical stimulation of DMV significantly increased the titratable acidity, the volume of gastric juice and the acid concentration. Similarly, electrical stimulation of either NA or DMV decreased the respiratory frequency and sinus bradycardia.

CONCLUSION: NA in rats can not control the secretion of gastric acid but the secretion of bicarbonate in gastric juice, while DMV controls the secretion of gastric acid.

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Key words: Rat; Nucleus ambiguus; Dorsal motor nuclei of vagus; Gastric acid; Gastric bicarbonate

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INTRODUCTION

The knowledge of brain stem nuclei controlling vagal parasympathetic outflow to the gastrointestinal tract has been derived principally from anatomic and physiological investigations of the dorsal motor nuclei of vagus (DMV). It was reported that electrical stimulation or lesion of DMV results in pronounced alterations in gastric motility and secretory function[1-4]. However, Kerr[2] showed that DMV is not the only origin of the vagal parasympathetic nerve innervating the stomach and vagal parasympathetic neurons might come from the nucleus ambiguus (NA). Studies indicate that the increased gastric motility in cold water-immersion stress rats is significantly inhibited, but there is no change in gastric acid secretion in rats pretreated with narceine or pentobarbital sodium[5,6]. These results suggest that the primary parasympathetic center innervating gastric motility and acid secretion consists of different nuclei. Moreover, recent morphological studies suggest that vagal parasympathetic neurons innervating the stomach are largely located in DMV and partly in NA[7-9]. So far, whether there are differences in the functions of DMV and NA in controlling gastric secretion remains unknown.

A great number of morphologic and physiological studies suggest that parasympathetic preganglionic neurons of the heart are located in DMV, NA and intermediate zone[10-13]. Previous experimental data have established that electrical stimulation of NA induces bradycardic responses, which can be abolished by injection of lidocaine into the rostral ventrolateral medulla[14,15].

It was reported that the medulla is the essential center of respiratory rhythm in mammals[16]. Respiratory neurons can be divided into two groups according to their positions: dorsal respiratory group and ventral respiratory group. NA is a component of ventral respiratory group[17]. A group of respiratory neurons in the rostral NA complex is involved in the generation of inspiratory and expiratory drives which enables spontaneous respiration[18]. The aim of this study was to investigate the effects of electrical stimulation of NA and DMV on gastric acid and bicarbonate secretion as well as respiratory frequency and heart rate.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 270-310 g were provided by

Table1 Effects of stimulation of NA on gastric H^+ and HCO_3^- secretion after pylorus ligation (mean ± SD)

	n = 8	Gastric juice (mL/3 h)	Titratable H^+ quantum (μmol/3 h)	H^+ concentration (mol/L)	HCO_3^- quantum (μmol/3 h)
Right	Control group	0.35 ± 0.18	47.6 ± 14.1	0.17 ± 0.08	11.0 ± 1.85
Right	Stimulus group	0.45 ± 0.24	43.1 ± 13.0	0.12 ± 0.09	15.8 ± 1.46[b]
Left	Control group	0.48 ± 0.23	46.6 ± 20.1	0.11 ± 0.05	10.8 ± 3.2
Left	Stimulus group	0.70 ± 0.30	58.0 ± 17.0	0.09 ± 0.03	17.2 ± 1.7[b]

[b]$P < 0.01$.

Table 2 Effects of stimulation of DMV on gastric H^+ and HCO_3^- secretion after pylorus ligation (mean ± SD)

	n = 8	Gastric juice (mL/3 h)	Titratable H^+ (μmol/3 h)	H^+ concentration (mol/L)	HCO_3^- quantum (μmol/3 h)
Right	Control group	0.62 ± 0.17	49.0 ± 12.5	0.08 ± 0.02	11.0 ± 3.4
Right	Stimulus group	1.00 ± 0.23[a]	109.0 ± 21.7[b]	0.11 ± 0.01[a]	10.2 ± 2.6
Left	Control group	0.67 ± 0.14	60.8 ± 14.3	0.09 ± 0.02	9.0 ± 3.6
Left	Stimulus group	0.97 ± 0.21[a]	115.8 ± 16.5[b]	0.13 ± 0.03[a]	11.5 ± 3.1

[a]$P < 0.05$, [b]$P < 0.01$.

Experimental Animal Center of Shandong University, China. The animals were individually housed in cages at 22 ± 2 ℃ with free access to food and water in a normal day/night cycle. All experiments were conducted according to the guidelines of the International Association for the Study of Pain[19] and every effort was made to minimize both the animal suffering and the number of animals used.

Experimental methods

The rats were intra-abdominally anesthetized with urethane (1 g/kg) and placed in a Jiangwan type I stereotaxic apparatus (made in China). The dorsal surface of the brain stem was exposed by limited occipital craniotomy. According to the coordination of the NA as defined by the atlas[20], an electrode (40-60 μm in diameter and 10-16 kΩ in resistance) was inserted vertically into the right or left side of the brain stem at a level 12.7 mm caudal to Bregma and 2.0 mm lateral to the midline, and to a depth of 9.6 mm below the dorsal surface. In the study on DMV, the electrode was inserted vertically into the right or left side of the brain stem at a level 13.8 mm caudal to Bregma and 0.7 mm lateral to the midline, and to a depth of 8.3 mm below the dorsal surface. Stimulation came from a BL-410 biological experimental system (Chengdu Taimeng Company, China). Stimulus parameters were 0.1 mA, 0.3 ms, 40 Hz and the stimulus duration was 10 min.

To study the effects of stimulation of NA and DMV on gastric secretion, pylorus ligation was used. A 2-mm tracheal cannula was inserted into the tracheal. The site between pylorus and duodenum was ligated. Three hours later, the site between cardia and esophagus was also ligated. The stomach was removed and gastric secretion was collected. Indices used to study gastric secretion were the volume of gastric juice, titratable H^+ quantum, H^+ concentration, HCO_3^- quantum. Values for titratable H^+ quantum were determined by back titration to pH 7.0 using 0.01 N NaOH. HCO_3^- secretion was determined as previously reported[21].

In the study on NA, we also used the method of esophagus perfusion to collect gastric secretion. A 2.5-mm tracheal cannula was inserted into the tracheal. A polyethylene tube (2 mm in diameter) was inserted into the esophagus to perfuse warm (37 ℃) normal saline at 2.0 mL/min. A 3-mm polyethylene tube was inserted into the stomach from the joint of cardia and duodenum to collect the gastric secretion. Base-line secretion values were determined from three consecutive 20-min values before stimulation. The three consecutive 20-min values were taken from beginning of the stimulation of NA to assess the change of secretion function. The index used to study gastric secretion was titratable H^+ quantum. Values for titratable H^+ quantum were determined by back titration to pH 7.0 using 0.01 N NaOH.

Respiration and electrocardiogram were continuously monitored using the BL-410 biological experimental system. Respiratory frequency and heart rate during stimulation were determined from the first 30 s of the stimulation period. Rectal temperature was monitored and maintained at 37-38 ℃ with a bulb.

Histological identification of stimulated sites

At the end of the experiments, the direct current of 1 mA was given to the stimulated site in the brain stem for 15 s. Then, 0.9% sodium chloride solution and 1% potassium ferrocyanide formalin solution were injected respectively into the aorta and out flowed from the right atrium. The brain was removed and fixed in a 10% formalin solution for 2-3 d. The brains were sectioned at 40 μm with a freezing microtome. Histological sections were stained with neutral red and examined microscopically. Only the results obtained when the tips of the stimulus electrode were just within the NA were used for statistical analysis.

Statistical analysis

Data were expressed as mean ± SD. The difference between two groups was evaluated by Student's *t* test. $P < 0.05$ was considered statistically significant.

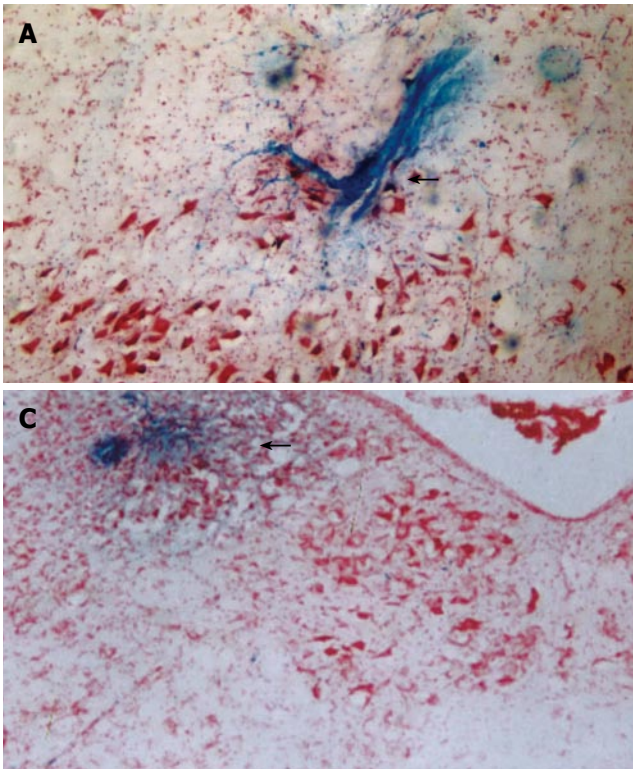
RESULTS

Histological identification of stimulated sites

The NA and DMV with their corresponding brain atlas are shown in Figure 1. The stimulated nuclei were identified according to the right atlas.

Effects of stimulation of NA and DMV on gastric H^+ and HCO_3^- secretion

Electrical stimulation of NA had no effects on the volume of gastric juice, titratable acidity and acid concentration, but elicited a pronounced increase in the total bicarbonate ($n = 8$, $P < 0.01$, Table 1). Electrical stimulation of DMV significantly increased the titratable acidity, the volume of gastric juice and acid concentration, with no change in bicarbonate quantum after pylorus ligation (Tables 1 and 2) and in titratable H^+ quantum after oesophagus perfusion (Figure 2).



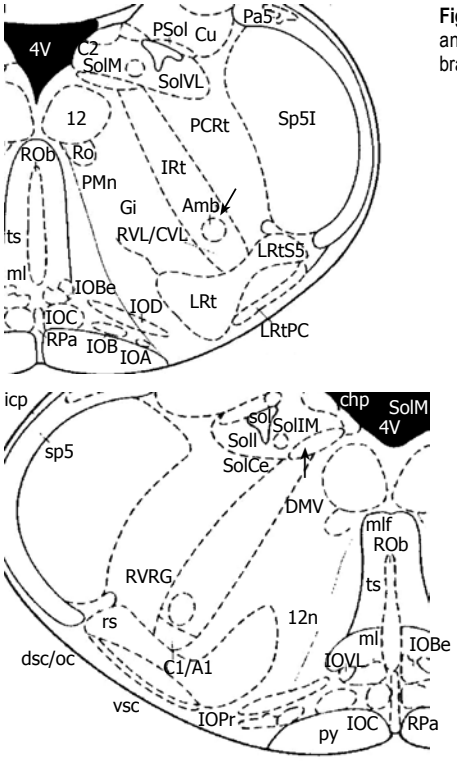


Figure 1 NA (**A**), DMV (**C**) and their corresponding brain atlas (**B**, **D**).

Table 3 Effects of stimulation of NA and DMV on respiratory frequency and heart rate (mean ± SD)

	n = 8	Respiratory frequency (times/min)	Inhibitory rate of respiratory frequency (%)	Heart rate (beats/min)	Inhibitory rate of heart rate (%)
Right NA	Control	91.2 ± 19.4		394.3 ± 70.1	
	Stimulation	34.9 ± 23.0[b]	51.5 ± 17.3	261.7 ± 77.1[b]	29.1 ± 8.9
Left NA	Control	101.8 ± 23.2		385.1 ± 37.6	
	Stimulation	45.6 ± 19.8[b]	64.7 ± 19.7	244.5 ± 44.3[b]	29.4 ± 12.3
Right DMV	Control	100.3 ± 7.6		402.8 ± 47.9	
	Stimulation	48 ± 11.5[b]	51.6 ± 12.3	268.5 ± 62.4[b]	34.2 ± 9.7
Left DMV	Control	102.8 ± 13.0		412.5 ± 33.3	
	Stimulation	60 ± 11.4[b]	41.3 ± 11.4	295.5 ± 52.2[b]	28.4 ± 10.7

[b]$P < 0.01$.

Effects of stimulation of NA and DMV on respiratory frequency and heart rate

Both respiratory frequency and heart rate were inhibited significantly after stimulation of NA and DMV ($n = 8$, $P < 0.01$). There were no significant differences in the inhibitory rate of NA and DMV (Table 3).

DISCUSSION

To our knowledge, the effects of NA and DMV on gastric H^+ and HCO_3^- secretion have not been examined up to now. In the present study, electrical stimulation of NA produced pronounced changes in HCO_3^- secretion but no changes in H^+ secretion. However, electrical stimulation of DMV produced pronounced changes in H^+ secretion but no changes in HCO_3^- secretion. The results of gastric H^+ secretion after stimulation of NA were consistent with those after pylorus ligation and

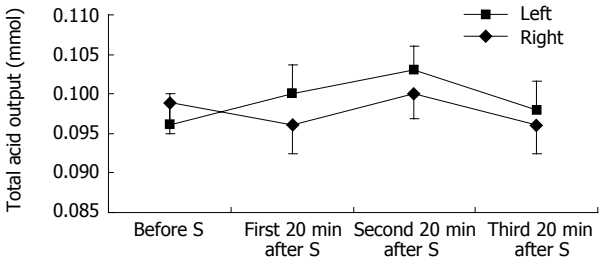


Figure 2 Effect of stimulation of NA on gastric acid secretion after oesophagus perfusion (S: stimulation).

esophagus perfusion. Pagani *et al*[22] found that electrical stimulation of cat NA produces no changes in gastric acid secretion. Morphological studies suggested that vagal parasympathetic neurons innervating the stomach are largely located in DMV and partly in NA[7,11]. These results indicate nervous fibers from NA and DMV innervate different cells of the stomach. NA innervates the nonacid-secreting cells and DMV innervates the acid-secreting cells. Our previous study also showed that gastric motility decreases significantly after stimulation of rat NA. Although this result is not consistent with that of Pagani *et al*[22], the gastric motility changes when NA is stimulated. All the results suggest the nervous fibers from NA mainly innervate gastric smooth muscles while the fibers from DMV innervate both gastric glands and gastric smooth muscles, which is consistent with the report of Kerr[2].

Morphologic and physiological studies suggest that parasympathetic preganglionic neurons of the heart are located in DMV, NA and intermediate zone[12], especially in NA. The NA, DMV, caudal ventrolateral medulla (CVLM) and lateral tegmental field (FTL) are located in the cardio-inhibitory area[23]. Electrical stimulation of NA and DMV

induces bradycardic responses, and the inhibitory rate of NA to the heart rate is not significantly different from that of DMV. Machado and Brody[15] also reported that electrical stimulation of NA induces bradycardic responses, which can be abolished by injection of lidocaine into the rostral ventrolateral medulla. NA efferent fascicles contain more large fibers (presumably B-type), whereas the DMV contains more fine caliber fibers (presumably C-type), and vagal control of the heart involves the convergence and integration of distinct NA and DMV projections within the cardiac plexuses[12]. In our study electrical stimulation of NA and DMV significantly decreased the respiratory frequency, and the inhibitory rate of respiratory frequency in NA was not significantly different from that in DMV. Rentero *et al*[24] investigated the activity pattern of cardiac motoneurons in rat NA using extra-cellular recordings and found that cardiac vagal motoneuron firing is modulated by the central respiratory cycle, showing peak activity during inspiration, which may be the mechanisms of respiratory sinus arrhythmias.

Both NA and DMV are the visceral motonucleus and located in the medullary visceral zone (MVZ), named "life center" which modulates the activities such as circulation, respiration, and digestion[25]. In our study, electrical stimulation of NA significantly increased HCO_3^- secretion, but induced no changes in H^+ secretion. In contrast, electrical stimulation of DMV significantly increased H^+ secretion, but induced no changes in HCO_3^- secretion. Similarly, stimulation of NA and DMV induced respiratory sinus arrhythmias. All these findings indicate that the effects of NA and DMV on gastric secretion, respiration and heart rate are mediated by the vagus.

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RAPID COMMUNICATION

Direct *ex vivo* analysis of dendritic cells in patients with hepatocellular carcinoma

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Abstract

AIM: To analyze the phenotype and function of dendritic cells (DC) from patients with hepatocellular carcinoma (HCC) in order to understand their role in this disease.

METHODS: Myeloid dendritic cells were enumerated in peripheral blood of HCC patients. CD80, CD83, CD86 and HLA-DR expression on naïve and stimulated myeloid dendritic cells from peripheral blood were analyzed. Myeloid dendritic cells were isolated from peripheral blood and their function was tested. Phagocytosis was analyzed using FITC-dextran beads, peptide specific stimulation, the capacity to stimulate allogeneic T cells and secretion of cytokines upon poly dI:dC was tested.

RESULTS: Myeloid dendritic cells were reduced in patients with HCC. No differences in CD80, CD83, CD86 and HLA-DR expression were found on naïve and stimulated myeloid dendritic cells from HCC patients and healthy controls. Normal phagocytosis or stimulation of peptide specific T cells was observed in contrast to an impaired allo-stimulatory capacity and a reduced IL-12 secretion.

CONCLUSION: Impaired IL-12 production of mDCs in patients could lead to an impaired stimulatory capacity of naïve T cells suggesting that IL-12 directed therapies may enhance tumor specific immune responses in HCC patients.

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Key words: Myeloid DC; Hepatocellular carcinoma; CD80; CD83; CD86

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world with a poor prognosis and limited survival in the majority of the patients[1, 2]. A number of different studies suggest that immunotherapeutical approaches will be successful for the treatment of this disease[3, 4]. Lymphocytic infiltration of the tumors is indicative of a better survival after surgical resection of the tumors[5]. Adjuvant treatment with activated lymphocytes has also been shown to increase tumor free survival after removal of the tumor[6]. Others and we identified potential tumor antigens for HCC[7-9], which can be used for vaccination purposes including peptide pulsed dendritic cells (DCs).

DCs are the most potent professional antigen-presenting cells (APC) unique in their ability to efficiently prime both $CD4^+$ and $CD8^+$ cytotoxic T cell responses following encounter with antigen and maturation[10]. Upon antigen uptake, DCs undergo the process of maturation and migration and upregulate adhesion and costimulatory molecules and secrete cytokines[11-13].

Two main subsets of DCs namely myeloid (mDCs) and plasmacytoid DCs have been described. These subsets differ in their origin, expression of phenotypic markers, route of activation and immunological activity[14]. $CD11c^+$ mDCs originate from myeloid bone marrow precursors and produce large amounts of IL-12 upon stimulation, which in turn promotes cell-mediated immune responses that are crucial for the detection and elimination of malignant cells[14, 15]. $CD11c^-$ pDCs, however, are of lymphoid origin, express high levels of IL-3 receptor-α chain and secrete less IL-12. A number of different antibodies have recently been generated, which allow for detection and isolation of these DC subgroups from peripheral blood

and other tissues[16]. The development of new DC specific antibodies[16] allows for direct analysis of DC numbers in peripheral blood and minimizes isolation or culture procedures for the enrichment of DCs that may induce the selection of particular cell subsets and phenotypic or functional modifications of DCs present in the blood[17].

Several studies have suggested that impaired function of DCs might be an important factor in the escape of the tumor from the immune control in cancer patients[18]. At least two mechanisms by which tumors can alter DC maturation and DC function are currently known. The first one affects the ability of hematopoietic progenitor cells to differentiate into functional DCs during the early stages of their maturation[19], whereas the second one affects the *in vitro* maturation of DCs from $CD14^+$ monocytes[20]. An understanding of the mechanisms of defects in DC function in different cancers will shed light on the role of DCs in tumor escape mechanisms[21]. Moreover, correction of DC dysfunction might be one possible option to enhance tumor-specific immune responses in HCC, which can be either used alone or in combination with immunotherapy to treat HCC.

This study investigates the frequency and function of circulating mDCs from patients with HCC directly *ex vivo*. mDCs were analyzed in detail for expression of costimulatory molecules and MHC class II surface expression directly *ex vivo* and after poly dI:dC stimulation. Most importantly, we have directly isolated mDCs from blood of HCC patients to test their function in four different functional assays: stimulation of allo-responses, stimulation of peptide-specific responses, phagocytosis and secretion of IL-10, IL-12 and TNF-α. We could clearly demonstrate that mDCs from patients with HCC displayed a normal phenotype, but were impaired in IL-12 secretion possibly leading to a decreased allostimulatory capacity observed in our study.

MATERIALS AND METHODS

Human subjects

Blood samples were collected from patients in the Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Germany. Written consent was obtained from all patients before blood and tumor sampling and the Ethics Committee of Hannover Medical School approved the study protocol. HCC was diagnosed according to the diagnostic guidelines of the European Association for the Study of the Liver (EASL)[22]. Serological tests (i.e., HCV RNA, hepatitis B surface antigen) were performed as previously described[7]. Liver cirrhosis and tumor staging were done according to Child-Pugh[23] and CLIP[24].

Quantification and phenotyping of mDCs

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood as previously described[7] and stained using the following antibodies: anti-CD1c-PE, anti-CD14-PE-Cy5, anti-CD19-PE-Cy5, anti-HLA-DR, anti-CD80-FITC, anti-CD83-FITC and anti-CD86-FITC (Pharmingen) or the corresponding isotype controls and analyzed on a fluorescence-activated cell sorter (FACS Calibur, Becton-Dickinson, Heidelberg, Germany). When indicated PBMCs were incubated for 4 h in the presence of poly dI:dC (Amersham Pharmacia). A total of 500 000 to 1×10^6 events were acquired for each sample on an FACS Calibur. 7-amino-actinomycin D (7-AAD) was used to exclude dead cells from analysis. Four-color analysis was performed using Cell Quest Software (Becton Dickinson, Heidelberg, Germany). Absolute numbers of DCs per ml of blood were calculated using the following equation: (% of gated DCs-% of isotype control) × absolute number of leukocytes.

Intracellular cytokine analysis

Intracellular FACS analysis was performed to analyze cytokine secretion by mDCs. PBMCs were isolated from peripheral blood and stimulated with poly dI:dC (Amersham Biosciences) for 48 h. Golgi-Stop (Pharmingen) was added during the last 6 h of stimulation. PBMCs were stained with a panel of antibodies to detect mDCs as described above, fixed, permeabilised and labeled with FITC-labeled antibodies against TNF-α, IL-10 and IL-12 and corresponding isotype controls (Caltag Laboratories). The evaluation of cytokine production was based on the percentage of cytokine expressing $CD1c^+$,$CD19^-$, $CD14^-$ cells.

Isolation of PBMC and CD1c+ DCs

PBMCs were isolated from heparinized blood by standard density gradient separation using Ficoll density gradient (Biochrom AG, Berlin, Germany). $CD1c^+$ cells were positively selected using a two-step immunomagnetic cell sorting procedure (MACS) following the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, $CD19^+$ cells were depleted from freshly isolated PBMCs followed by positive selection of $CD1c^+$ cells. Purity of $CD1c^+$ DC was controlled by flow cytometry and found to be > 85%.

Quantification of endocytosis by freshly isolated mDCs

Measurement of FITC-dextran uptake was performed as previously described[25]. In brief, freshly isolated mDCs were suspended in culture medium and FITC-dextran (MW 40 000) (Molecular Probes, Leiden, The Netherlands) was added at a final concentration of 1.0 mg/mL and incubated at 37 ℃ for 30 min. The DCs were washed four times with cold PBS containing 1% fetal calf serum and the incorporation of FITC-dextran was analyzed using FACScan. The incorporation of FITC-dextran at 4 ℃ was used as control. The data are shown as fluorescence intensities.

Mixed lymphocyte reactions using freshly isolated CD1c positive DCs

Allogeneic PBMCs (1×10^5) from a healthy donor were resuspended in complete RPMI medium (Gibco BRL, Eggenstein, Germany) supplemented with 2% human AB serum, plated with indicated numbers ($10\text{-}10^4$) of $CD1c^+$ DCs in 96 well plates (Greiner, Frickenhausen, Germany) and incubated at 37 ℃ in a humidified incubator contain-

ing 50 ml/L CO_2. Phytohaemagglutinin 1 μg/mL (Sigma Aldrich, Munich, Germany) was added to PBMC in separate wells as a positive control. After 5 d, 1 μCi/well [3H]-thymidine (Amersham Biosciences, Freiburg, Germany) was added and plates were harvested after 16 h and harvested onto filters using an automated cell harvester (Wallac, Turku, Finland). The amount of [^{3}H]-thymidine incorporated into DNA was measured by a beta-counter (Wallac, Turku, Finland), and the results were expressed as mean counts per minute (cpm). A stimulation index (SI) was calculated for each donor by dividing the mean cpm of the replicates of DC stimulated PBMC by that of the control cultures (PBMC incubated in medium in the absence of DCs).

Analysis of antigen-specific stimulatory potential using a peptide specific T cell line

mDCs (1×10^5) were incubated with the M1 peptide or an irrelevant peptide control for 1 hour. After 1 hour, cells were washed and incubated with 1×10^5 M1 specific T cells. In parallel, T cells were stimulated with T2 cells pulsed with the M1 peptide or a negative control. After 20 hours supernatants were harvested and tested for GM-CSF release by ELISA (Pharmingen).

Statistical analysis

Kruskall-Wallis' analysis of variance and Student's *t* test were used to assess differences between the different study cohorts. Differences with a *P* value of < 0.05 were considered statistically significant.

RESULTS

Detection and quantification of mDCs in peripheral blood

Peripheral blood from a total of 25 patients with HCC was analyzed. Patients' and control group clinical characteristics are shown in Tables 1 and 2. Using a CD1c specific antibody, mDCs were detected in peripheral mononuclear cells and the frequency was determined for every individual subject tested. As shown in Figure 1, the median frequency of mDCs was significantly ($P < 0.001$) decreased for HCC patients (1.5 ± 0.24 mDCs/μL), compared with 23 healthy controls (3.14 ± 0.41 mDCs/μL). In order to exclude that the observations made was only due to chronic liver disease but not due to the presence of HCC, we have also analyzed mDCs from different control groups such as patients with chronic HBV and HCV infection, non-viral liver cirrhosis as well as patients with metastasis from colorectal carcinoma. However, significantly lower mDC frequencies were found in blood from patients with HCC, when compared to patients with chronic HBV infection (2.03 ± 0.52 mDCs/μL), non-viral liver cirrhosis (1.95 ± 1.2 mDCs/μL) and colorectal liver metastasis (1.48 ± 0.61 mDCs/μL) (Figure 1A). In addition, direct comparison of HCC patients with and without chronic viral hepatitis revealed no significant differences as shown in Figure 1B.

Phenotypic analysis of circulating mDCs in peripheral blood before and after in-vitro stimulation

mDCs from patients with HCC and healthy controls were further analyzed for the expression of costimulatory molecules and MHC class II by FACS analysis directly *ex vivo* (Figure 2A). No significant differences were found for the expression of CD80 (HCC patients 0.9 ± 0.05 *vs* healthy 1.0 ± 0.04), CD83 (HCC 1.54 ± 0.38 *vs* healthy 2.2 ± 0.51), CD86 (HCC 36.5 ± 9.54 *vs* healthy 34.7 ± 9.57) and HLA-DR (HCC 83.2 ± 10.1 *vs* healthy 89.5 ± 15.2). Next, mDCs, which are known to express TLR3[26], were stimulated with poly dI: dC and expression of CD83, CD86 and MHC class II was analyzed. As shown in Figure 2B, poly dI:dC stimulation induced a strong up-regulation of CD83 on mDCs. However, there was no difference in CD83 up-regulation between mDCs from HCC patients and healthy controls (6.58 *vs* 8.09). Similar results were found when stimulated mDCs from HCC patients and healthy controls were analyzed for up-regulation of CD86 (HCC 3.53 *vs* healthy 4.09) and HLA-DR (HCC 1.88 *vs* healthy 1.8), indicating no difference in the ability of mDCs to mature after poly dI:dC stimulation.

Table 1 Clinical characteristics of HCC patients analyzed in Figure 1 A and B

Age (yr)	Cause	Sex	Child score	AFP (μg/L)	CLIP score	mDCs (cells/μL)
58	Ethanol	M	A	419	1	0.76
61	Ethanol	M	A	5	1	1.46
65	Ethanol	M	A	380	1	1.67
67	Unknown	M	B	5	2	2.35
55	Ethanol	M	B	3399	3	2.02
70	Ethanol	M	C	5829	4	0.16
76	HBV	M	B	8	2	0.22
67	HBV	M	C	126	3	0.99
49	HBV	M	A	3845	1	0.54
55	HBV	M	B	5	0	4.93
46	HCV	M	B	32	1	1.17
69	HCV	M	A	8803	2	2.09
64	HCV	F	B	2458	4	0.28
63	HCV	M	A	5	3	1.03
70	Ethanol	F	B	6	3	0.82
72	Unknown	M	A	64	2	3.95
63	Ethanol	M	A	9	0	2.28
56	HCV	M	B	10	1	1.40
8	Ethanol	M	A	155509	3	0.55
64	HCV	F	A	6	3	2.04
67	None	M	n/a	18	2	1.37
55	HCV	M	B	33	1	1.07
71	HBV	M	A	10	2	0.71
61	Ethanol	M	B	455	3	2.63
70	HCV	F	A	3462	1	1.19

Table 2 Clinical characteristics of control groups with HCC in Figure 1 A

Group	Age (Mean, yr)	Liver cirrhosis
Healthy	64	None
HCV	54	None
HBV	45	None
Liver cirrhosis	46	Child A and B
Liver metastasis	64	None

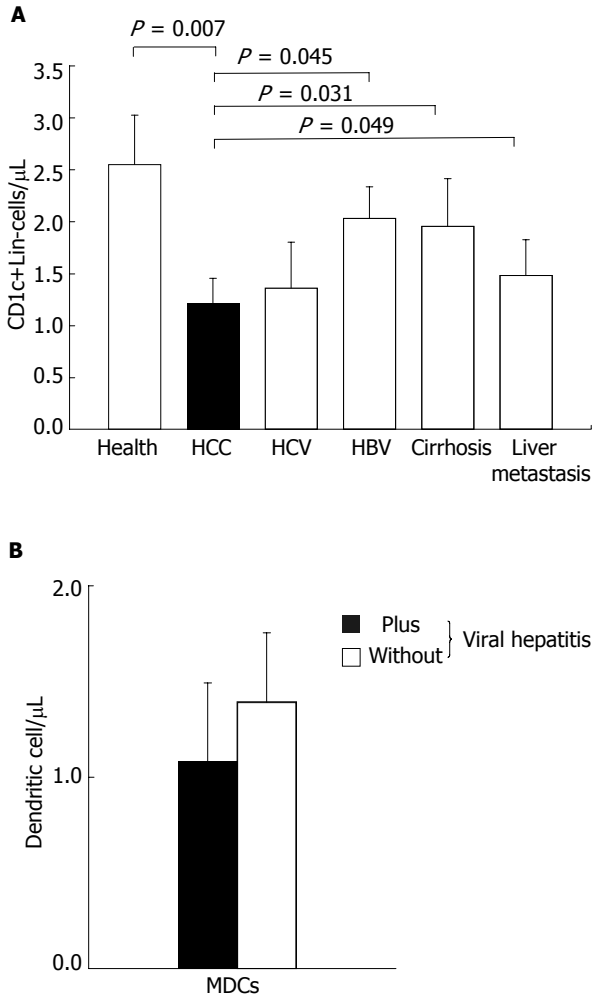


Figure 1 Quantification of dendritic cell subtypes. **A**: Analysis of different patient groups; **B**: Comparison of HCC patients with or without chronic viral hepatitis.

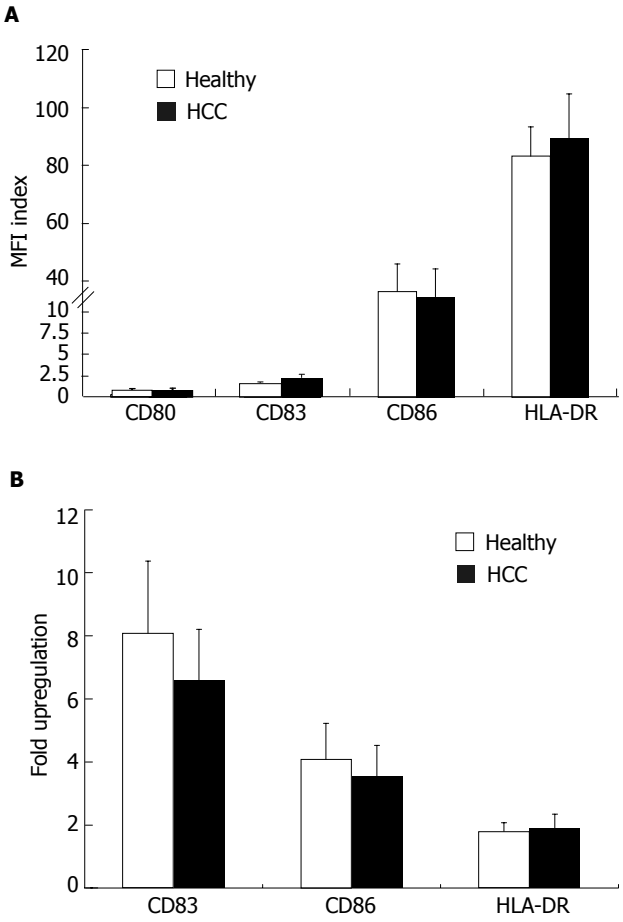


Figure 2 Phenotypic analysis of myeloid dendritic cells in peripheral blood.

FITC-dextran uptake by mDCs from HCC patients and healthy controls

Using CD19 depleted cells, we were able to isolate CD1c$^+$ mDCs from peripheral blood and analyze their function directly *ex vivo* and without *in vitro* stimulation or maturation. This procedure resulted in > 85% pure mDC populations (data not shown), avoided *in vitro* artifacts and therefore mimicked closely the *in vivo* situation. First, endocytosis ability of freshly isolated mDCs was measured using FITC dextran beads. FITC dextran uptake was compared between mDCs from HCC patients and healthy controls. As shown in a representative experiment (Figure 3A), phagocytosis could easily be followed over time with an increase of mean fluorescence intensity (MFI) from 6.3 to 14.6, 19.7 and 27.7 at 0, 7, 15 and 30 min respectively. Figure 3B shows the relative increase of MFI for the two groups after 7, 15 and 30 min. No significant difference was seen when endocytosis by mDCs from HCC patients and healthy donors was compared.

mDCs can stimulate antigen-specific T cells

Since mDCs from HCC patients and healthy controls could take up dextran beads equally well, we next analyzed the antigen presenting capacity of these cells. To assess the ability of mDCs to stimulate antigen-specific CD8$^+$ T cells, mDCs from HLA-A2 positive healthy donors and HCC patients were used to stimulate T cells specific for a common recall antigen, the Influenza matrix peptide M1[27]. mDCs were pulsed with M1 peptide and an irrelevant peptide and used to stimulate M1-specific CD8$^+$ T cells. T2 cells pulsed with the same peptides were used as control for every experiment in parallel. Cytokine secretion by M1 specific CD8$^+$ T cells was analyzed in the cell supernatants. Influenza M1 specific T cells secreted 306 pg/mL GM-CSF when mDCs from HLA-A2 positive donors were used (Figure 4) and slightly lower GM-CSF was released when M1 specific T cells were stimulated with mDCs from HCC patients (159 pg/mL). However, this difference was not significant, since the T2 stimulation (as a positive control) was also slightly different between the two experiments (Figure 4). As expected, no peptide specific GM-CSF secretion was found when mDCs were used from an HLA-A2 negative donor (data not shown).

The allo-stimulatory capacity of mDCs from HCC patients is impaired

To test the allostimulatory potential of mDCs from HCC patients, we studied their function in an allogeneic mixed

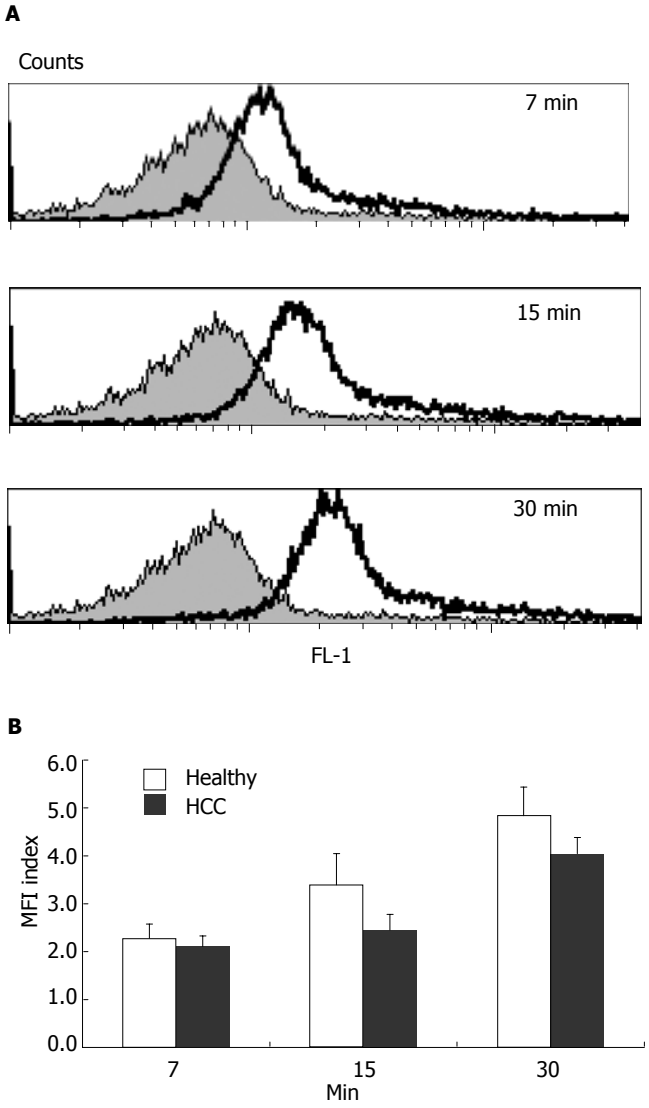


Figure 3 FITC-dextran uptake by mDCs.

lymphocyte reaction (MLR). mDCs were isolated from peripheral blood of HCC patients and healthy controls. PBMCs from a third healthy donor were used as responder cells. Figure 5 summarizes the average of eight HCC patients and six healthy donors. mDCs from HCC patients were clearly less efficient in inducing T cell proliferation than mDCs from healthy donors. For all the stimulator concentrations tested (10-10^4), a significant reduction in allostimulatory capacity of mDCs was seen ($P < 0.05$). Ten thousand mDCs from HCC patients stimulated allogeneic T cells almost 3-fold less than mDCs from healthy donors with a stimulation index of 13 *vs* 35. When 10 fold fewer mDCs were used, still a significant difference in allostimulatory function was seen. It has been shown that patients with chronic HCV infection have impaired function of circulating mDCs[28]. Therefore, we compared the allostimulatory potential of mDCs from patients with and without chronic HCV infection to mDCs from healthy controls. Our analysis clearly demonstrated that not only patients with chronic HCV infection and HCC but also those patients without chronic hepatitis have impaired circulating mDCs in peripheral blood (Figure 5B).

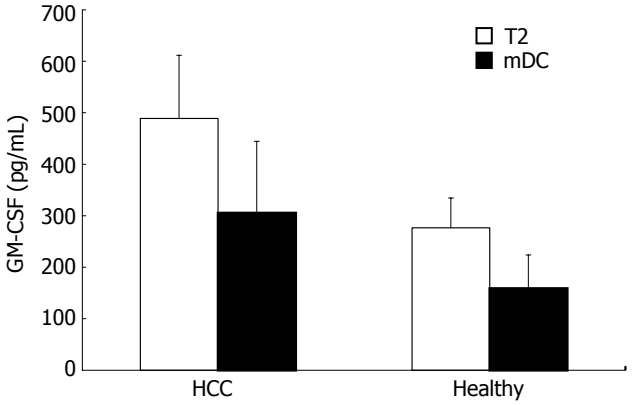


Figure 4 Peptide-specific stimulation by mDCs.

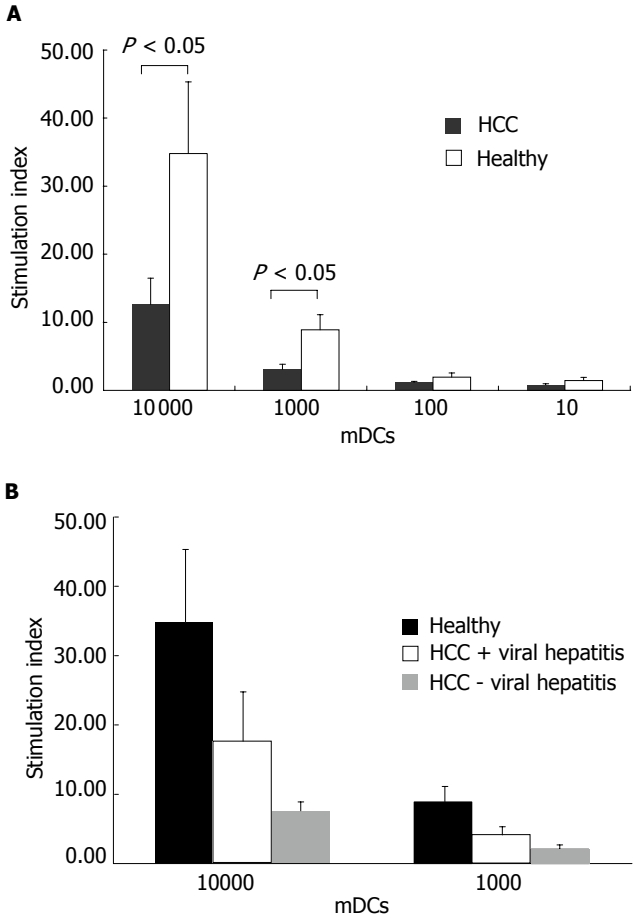


Figure 5 Impaired allogeneic MLR stimulation by mDCs from HCC patients. **A**: Stimulation index of mDCS from HCC patients and healthy controls; **B**: Comparison of stimulation index of mDCs derived from healthy controls and patients with or without chronic viral hepatitis.

Impaired cytokine production by mDC from HCC patients

We next analyzed the expression of cytokines by mDCs, since a perturbation of the cytokine network has been previously reported in patients with neoplasms including HCC[29-32]. Therefore, we analyzed the expression of IL-10, TNF-α and IL-12 by mDCs in HCC patients. PBMCs from healthy donors and HCC patients were stimulated for 48 h with poly dI: dC and analyzed for cytokine secretion by intracellular cytokine staining.

As shown in Figure 6, no significant differences for

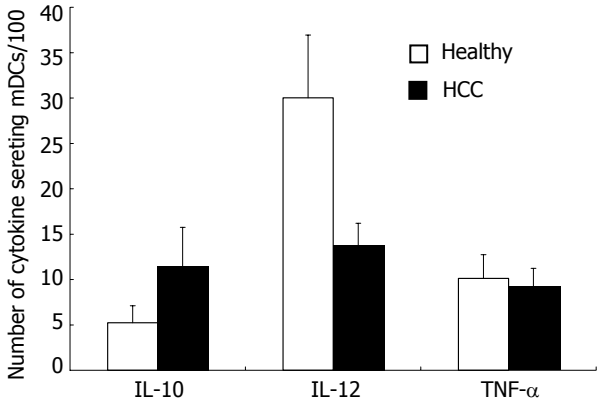


Figure 6 Impaired cytokine production by mDCs from HCC patients.

TNF-α and IL-10 production by mDCs were found. In contrast, the percentage of mDCs expressing IL-12 upon poly dI:dC stimulation was significantly lower in HCC patients (30.2) than in healthy controls (13.76, $P < 0.01$).

DISCUSSION

Until today treatment options for HCC are limited to surgery and local ablative therapy[33, 34]. Therefore, much emphasis has been put on understanding the development of HCC and investigating new therapeutic options, of which one is immunotherapy[3]. Previously, we have shown that more than 50% of HCC patients develop tumor specific immune responses against a well-defined tumor antigen[7]. However, in most patients analyzed, tumors progressed in spite of tumor specific humoral and cellular immune responses. Therefore, we are interested in investigating possible immunological mechanisms, which might lead to suppression of antigen-specific T cells in HCC patients. Recently, we have shown that $CD4^+CD25^+$ regulatory T cells are increased in both peripheral blood and tumor of HCC patients[35]. However, it is highly likely that a combination of regulatory T cells along with other factors is responsible for a defective anti-tumor immune response seen in HCC patients. Impaired function of professional antigen presenting cells has been suggested as a possible mechanism to promote tumor escape from immune control[21].

A number of different studies have analyzed the function of monocyte derived DCs in different liver diseases including HCC[25, 36, 37]. However, recent studies have shown that impaired function of monocyte derived DC might be the result of inefficient culture conditions rather than of patients' diseases[38]. Therefore we have performed our analysis on directly *ex vivo* isolated DCs avoiding *in vitro* manipulations. In order to verify that the findings made are not simply due to underlying liver diseases such as chronic viral hepatitis or liver cirrhosis, which can be found in the majority of patients with HCC[2], different control groups such as patients with HBV and HCV infection, liver cirrhosis and liver metastasis from colorectal cancer were included in the study for comparison. In addition, HCC patients with and without chronic viral hepatitis were compared with each other. Consistent with published data by others, we have observed a decreased number in circulating mDCs[31], which is an unlikely reason for impaired T cell responses seen in patients with different type of tumors including HCC[21]. Therefore, the main focus of our study was to investigate the function of mDCs isolated from patients with HCC. Professional antigen presenting cells pick up antigens, process and present them to T cells and cause potent T cell stimulation by expression of appropriate cytokines and costimulatory molecules[10]. Therefore, we performed a detailed step-by-step functional analysis of *ex vivo* isolated non-manipulated mDCs from patients with HCC and compared them to mDCs from healthy controls.

Interestingly, most functional assays performed showed no differences between mDCs from HCC patients and healthy controls such as expression of costimulatory molecules, most cytokines analyzed, antigen uptake and peptide presentation. However, when mDCs from HCC patients or healthy controls were tested to stimulate allogeneic PBMC, mDCs from HCC patients were clearly less potent stimulators. Similar results have been observed for patients with other tumors[29] and non-malignant liver diseases[39]. In addition, the observations made were not due to underlying chronic viral hepatitis, since mDCs from HCC patients with or without chronic viral hepatitis were less potent stimulators. However, the exact mechanism for this observation has not been elucidated. Because no differences were found for the MHC class II, CD80, CD83 and CD86 expression on mDCs, we extended our analysis for other potential factors leading to reduced stimulatory capacity of DCs.

Cytokines secreted by DC have been shown to have an important regulatory role in activation of the immune response. Therefore, we analyzed the expression of three different cytokines; TNF-α, IL-10 and IL-12 by mDCs upon stimulation. Although, we could not detect differences in the number of TNF-α or IL-10 expressing mDCs, significant differences were found in the numbers of IL-12 producing mDCs between HCC patients and healthy donors. IL-12 has been shown to play an anti-tumor role *in vivo*[32, 40]. An imbalance in the cytokine expression pattern as shown by low levels of IL-12 and normal or higher levels of IL-10 has been seen in other cancer patients[32, 41]. Our data suggests that reduced IL-12 expression of mDCs from HCC patients could lead to an impaired T cell stimulatory effect, which could not only account for a reduced allogeneic T cell response but also further account for an impaired general immune response.

Although, we have not been able to demonstrate this in our study due to limitations in the amount of blood that could be obtained from HCC patients, one could postulate that exogenous IL-12 might reverse impairment of DC function seen in our study. Indeed a number of clinical studies have seen promising results treating HCC patient with thalidomide in selected cases[42, 43]. Moreover, it has been shown that thalidomide might lead to an enhanced IL-12 secretion by DC s *in vitro*[44].

In summary, direct evaluation of DCs from HCC patients without *in vitro* manipulation has allowed us to

clearly demonstrate a decrease in the frequency of mDCs in peripheral blood of HCC patients and more importantly a functional defect of these cells leading to impaired stimulation of allogeneic T cells. Decreased IL-12 production by mDCs might be a possible cause of this impairment, which suggests that IL-12 directed therapies could reverse this phenomenon and should be further investigated in the context of immunotherapeutic approaches to the treatment of HCC.

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RAPID COMMUNICATION

Investigation of *HER-2* codon 655 single nucleotide polymorphism frequency and c-ErbB-2 protein expression alterations in gastric cancer patients

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Abstract

AIM: To investigate both whether the risk of gastric cancer is associated with the Ile/Val single nucleotide polymorphism (SNP) of human epidermal growth factor receptor-2 (*HER-2*) transmembrane domain-coding region at codon 655 and the suggested existence of *HER-2* expression in gastric cancer cases in a Turkish patient group.

METHODS: Polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) strategy was used to analyze the presence of *HER-2* SNP at codon 655. c-erbB-2 expression pattern was analyzed by immunohistochemistry. The results were compared between gastric carcinoma group and chronic gastritis group, as well as between clinicopathological parameters and carcinoma.

RESULTS: Results showed that Ile/Val genotype accounted for 20% within the Turkish gastric carcinoma group, and none in chronic gastritis group, and this genotyping was associated with stage IV gastric cancers (P = 0.04). Positive membranous *HER-2* immunoreactivity, on the other hand, accounted for 24% within the Turkish gastric carcinoma group and none from chronic gastritis cases; further, it was correlated with intestinal type carcinomas (P = 0.007), and stage III-IV carcinomas (P = 0.004).

CONCLUSION: These observations imply that the tested *HER-2* SNP may participate in the development and progression of gastric cancer. Thus, after confirming these results with large sample groups, *HER-2* codon 655 SNP and/or c-erbB-2 overexpression may also be used as a poor prognostic indicator for gastric carcinomas.

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Key words: Gastric carcinoma; *HER-2*; c-erbB-2; Single nucleotide polymorphism; Immunohistochemistry

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INTRODUCTION

Gastric adenocarcinoma is the second leading cause of cancer-specific mortality worldwide and efforts intended for prevention and early detection of gastric carcinoma are effective and yet important[1]. Despite various treatments such as gastrectomy with extensive lymphadenectomy and surgery combined with chemotherapy, the control of gastric cancer at advanced stages is still challenging.

Molecular genetic analyses have indicated that transformation of normal epithelial cells into a cancer is a multi-step process associated with the progressive accumulation of abnormalities in DNA repair genes, tumor suppressor genes, oncogenes, cellular growth factors, surface receptors, and cellular adhesion molecules[2]. Structural and functional alterations of human epidermal growth factor receptor-2 (*HER-2*) also known as c-ErbB-2/neu proto-oncogene have been reported in different steps of carcinogenesis including initiation, promotion and progression[3]. *HER-2* is a member of the epidermal growth factor (EGF) receptor family located at chromosome 17q21 that encodes a 185-kDa transmembrane glycoprotein with tyrosine kinase activity[4-6]. It is localized to the cell membrane and binding of extracellular growth factor ligands to HER-2 leads to the formation of dimers with another molecule of HER-2 or with HER-1 (EGFr), HER-3 or HER-4. This dimerization results in phosphorylation of tyrosine residues on the cytoplasmic domain of the receptor and subsequent activation of intracellular signaling pathways that regulate diverse biologic responses, including proliferation,

differentiation, cell motility, and survival[7]. Overexpression of c-erbB-2 has frequently been reported in various cancer types including breast, ovary, lung, salivary gland, colon, prostate and pancreatic and has been suggested to be an indicator of poor prognosis[8,9]. *HER-2* DNA amplification is suggested to be the principal mechanism of *HER-2*/neu protein activation and is nearly always accompanied by its protein overexpression in breast cancer. It has been also reported c-erbB-2 overexpression in 10% to 38% of gastric cancer cases using immunohistochemical, Southern blot, and fluorescent *in situ* hybridization analyses that suggest differences in c-erbB-2 expression among subtypes of gastric cancer[10-13]. However, controversy continues in terms of correlation between the HER-2 protein expression and gastric cancer prognosis. There have been reports correlating the lymph node metastasis and/or liver metastasis with HER-2 expression[14-17], whereas others showed no correlation between clinicopathologic findings and HER-2 expression[18,19]. In addition, it has also been shown that HER-2 expression is fewer than 10% in diffuse adenocarcinomas[16,17]. The use of trastuzumab, a function blocking monoclonal antibody against the extracellular domain of HER-2, as a treatment option in HER-2-overexpressing advanced breast cancer patients underlines once again the importance of this protein and its downstream signaling cascade(s) in the pathogenesis of above-mentioned cancers.

Presence of a single nucleotide polymorphism (SNP) in the transmembrane coding region of the *HER-2* gene at codon 655, encoding either isoleucine (Ile: ATC) or valine (Val: GTC), has been reported in different cancer types[12,13]. Changing the existing isoleucine (Ile: ATC) to valine (Val: GTC) at codon 655, suggests an increased dimerization, autophosphorylation of HER-2 and tyrosine kinase activity, which may cause the transformation of cells[16]. Some of the reports have revealed that the presence of Ile/Val SNP is associated with increased risk of breast cancer development[20]; on the other hand, others have shown that this correlation is controversial[21-24]. One reason for these contradictory results might be the substantial differences in genetic polymorphism in the *HER-2* codon 655 between ethnic groups.

In this study, the frequency of *HER-2* codon 655 polymorphism, resulting in an A to G transition (Ile655Val) and alterations in c-erbB-2 protein expression have been investigated in Turkish gastric cancer patients in association with the clinicopathologic parameters.

MATERIALS AND METHODS

Materials

The specimens at Pamukkale University Department of Pathology archive have been used and paraffin-embedded tissues of 25 gastric carcinomas (8 with diffuse type, 16 with intestinal type, i.e., 5 with well, 4 with moderately, 7 with poorly differentiated adenocarcinoma, classified according to Lauren classification and 1 with adenosquamous type) were selected as the study group in this study (Table 1). All carcinomas were staged according to UICC criteria, graded according to WHO criteria, and histopathologically typed according to Lauren classification. Paraffin-embedded tissues of 18 chronic gastritis cases selected from the same collection served as the control group.

Table 1 Clinicopathological details of gastric carcinoma cases

		Patients (*n*)
Gender	Male	15
	Female	10
Age (yr)(mean ± SD)	35-80, (58.8 ± 12.8)	
Histologic type (Lauren)	Intestinal	16
	Diffuse	8
	Adenosquamous	1
Histologic type (WHO)	Well	7
	Moderatelly	5
	Poorly	4
Serosa invasion	Positive	19
	Negative	1[1]ND
Nodal involvement	Positive	14
	Negative	6[1]ND

[1] Not Determined: 5 cases were endoscopic biopsy samples so cannot be determined.

DNA extraction and mutation analysis

DNA extraction from paraffin-embedded tissues was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden-Germany) according to the manufacturer's protocol. SNP in *HER-2* transmembrane segment involving codon 655 was analyzed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) strategy[25]. The 148 bp transmembrane segment of *HER-2* was PCR-amplified using the gene specific primers, i.e., forward (*HER-2*/F) 5'-AGAGCGCCAGCCCTCTGACGTCCAT-3', and reverse (*HER-2*/R) 5'-TCCGTTTCCTGCAGCAGTCTCCGCA-3'[25]. All PCR amplifications were performed in a total volume of 50 μL containing 10 μL of extracted DNA, 20 pmol/L of each forward and reverse primer, and 25 μL of HotStarTaq Master Mix [containing 2.5 units of HotStarTaq DNA polymerase, 1 × PCR buffer with 1.5 mmol/L $MgCl_2$, and 200 μmol/L of each dNTP (Qiagen, Hilden-Germany)]. Thermal cycling conditions for the PCR are as follows: initial activation of HotStarTaq DNA polymerase at 95 ℃ for 15 min, followed by 35 cycles of 94 ℃ for 0.5 min, 62 ℃ for 0.5 min, 72 ℃ for 1 min, and a final extension of 72 ℃ for 10 min. PCR products were subjected to electrophoresis on a 2% ethidium bromide (Et-Br) stained agarose gel and visualized under UV for the control of their specificity and integrity.

For RFLP analysis, 5 μL of each PCR product was digested with *Bsm*AI (New England Biolabs, Ipswich, MA-USA) at 55 ℃ for 2 h, and enzyme was heat-inactivated at 80 ℃ for 20 min. *Bsm*AI digestion gave fragments of 116 bp and 32 bp for the Val (GTC) allele and a single 148 bp fragment for the Ile (ATC) allele[11]. Fragments digested with *Bsm*AI were subjected to electrophoresis on a 3% molecular screening grade agarose gel (Roche Diagnostics, GmbH, Mannheim-Germany), stained with Et-Br and visualized under UV light.

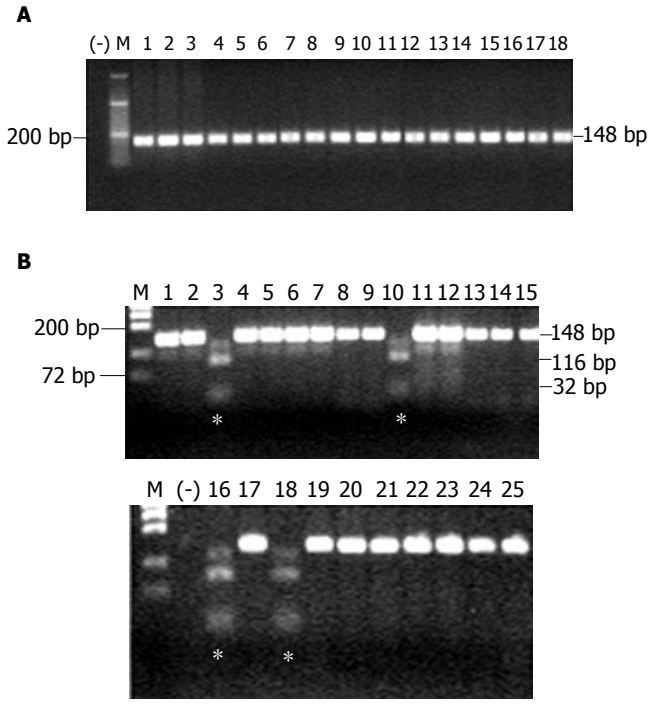


Figure 1 SNP analysis in **(A)** chronic gastritis group, and **(B)** gastric carcinoma group. The SNP in codon 655 of *HER-2* was detected in patients No 3, 10, 16, and 18 of gastric carcinoma group (*). M, molecular weight marker; (-), negative control for PCR; bp, base pair.

Table 2 Summary of *HER-2* SNP presence and c-erbB-2 expression in gastric carcinoma cases

Patient No	Classification according to Lauren	Stage	Ile/Val genotype	Immunohistochemical expression score for c-erbB-2
1	D	4	-	0
2	D	4	-	0
3	I	4	+	1
4	D		-	0
5	D		-	0
6	D	4	-	0
7	D	3	-	0
8	D	4	-	0
9	I	4	-	0
10	I	4	+	3
11	D	3	-	0
12	I	4	-	2
13	I	2	-	1
14	I	4	-	0
15	I	4	-	1
16	I	4	+	0
17	I	1	-	1
18	I	4	+	3
19	I		-	1
20	I	4	-	2
21	I	2	-	2
22	I		-	0
23	I		-	2
24	I	3	-	0
25	Other	4	-	0

D: diffuse type; I: intestinal type; (-): negative; (+): positive.

Immunohistochemistry

Formalin fixed, paraffin-embedded tumor-rich representative tissues were used for immunohistochemical staining. Reactions were performed with anti-c-erbB-2 polyclonal antibody (DAKO, Glostrup-Denmark) using the streptavidin biotin peroxidase method along with DAB as chromogen by Ventana-NexES IHC automatic immunostainer. Staining patterns were analyzed semiquantitatively and scored in the range of 0 to 3, score 0: Less than 10%; score 1: More than 10% of tumor cells with partial membranous staining; score 2: More than 10% cells with moderate membranous staining; score 3: More than 10% cells with strong membranous staining.

Statistical analysis

The data were analyzed by the Chi-Square test. Differences at $P < 0.05$ were considered to be significant. Statistical analyses were performed with SSPS software, Version 11.0.

RESULTS

HER-2 PCR-RFLP analysis showed that none of the 18 chronic gastritis cases had the SNP in *HER-2* gene at codon 655 (Figure 1A), whereas 4 of 25 gastric carcinoma cases (20%) had the Ile/Val genotype (Figure 1B, Table 2). The difference between these two groups were significant ($P = 0.04$). All 4 mutation positive cases were stage IV in terms of clinicopathologic parameters (Table 2). However, presence of this SNP did not show any correlation with the age, gender, histological type (according to Lauren), serosal invasion, lymph node metastasis, and distant metastasis.

Positive membranous c-erbB-2 immunoreactivity (scores 2-3), on the other hand, was detected in 6 of 25 carcinoma cases (24%), i.e., 4 cases with score 2 (16%) and 2 cases with score 3 (8%) (Figure 2A and B, Table 2), whereas 5 cases showed score 1 staining (20%) and 14 cases showed no staining at all (56%, Table 2). Results from chronic gastritis cases revealed no positive membranous c-erbB-2 immunoreactivity, i.e., 4 cases had score 1 staining (19%) (Figure 2C) and 17 cases had no staining at all (81%). When the results from carcinoma group were compared with clinicopathologic parameters, positive membranous c-erbB-2 immunoreactivity was correlated with intestinal type carcinomas ($P = 0.007$), and stage III-IV carcinomas ($P = 0.004$). However, no correlation with the age, gender, serosal invasion, lymph node metastasis, and distant metastasis was observed.

DISCUSSION

The aim of this study was to investigate both whether the risk of gastric cancer is associated with the Ile/Val SNP of *HER-2* transmembrane domain-coding region at codon 655 and the suggested existence of c-erbB2 expression in gastric cancer cases in a Turkish patient group. Results showed that Ile/Val genotype accounted for 20% of the Turkish gastric carcinoma group ($P = 0.04$), and this genotype was associated with stage IV gastric cancers. However, it did not show any correlation with the age, gender, histological type (according to Lauren classification), serosal invasion, lymph node metastasis, and distant metastasis. Positive membranous c-erbB-2 immunoreactivity, on the other hand, accounted for 24% of the Turkish gastric carcinoma group and it was found to be correlated with intestinal type carcinomas ($P = 0.007$), and stage III-

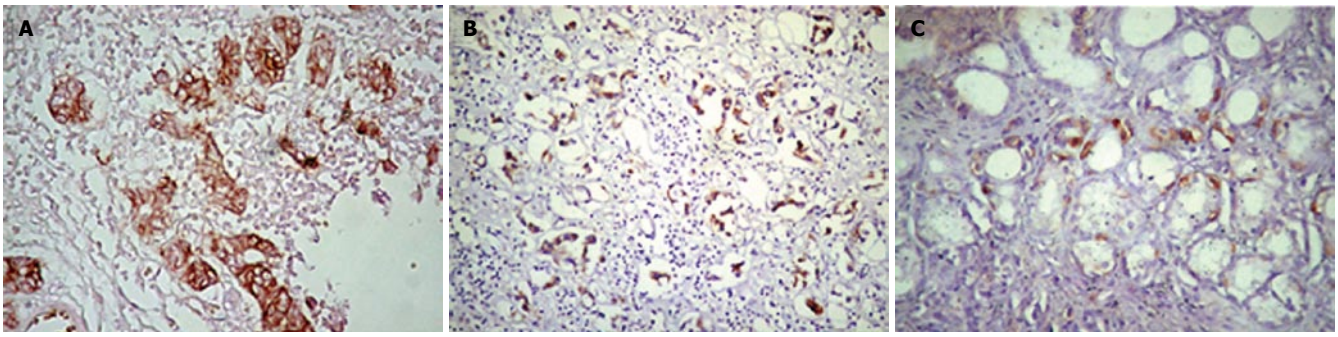


Figure 2 Representative images of HER-2 immunoreactivity in **(A)** moderately differentiated intestinal type adenocarcinoma (score 3), **(B)** poorly differentiated intestinal type adenocarcinoma (score 3), and **(C)** chronic gastritis (score 1).

IV carcinomas ($P = 0.004$). These observations imply that the tested *HER-2* SNP may participate in the development and rapid progression of gastric cancer. However, further larger sample studies particularly containing early stage gastric carcinoma patients, are needed to confirm the presence of SNP and/or c-erbB-2 overexpression may also be used as a poor prognostic indicator for gastric carcinomas.

HER-2 encodes a receptor tyrosine kinase functioning as a growth regulatory protein and a cell motility factor[26]. Alterations of *HER-2* have been implicated in the carcinogenesis and are frequently observed in a variety of tumors[24, 27-29]. The molecular mechanism of the association between the Ile/Val SNP at codon 655 of *HER-2* and cancer has not been fully understood; however, it has been suggested that Ile/Val SNP at codon 655 of *HER-2* results in increased dimerization, autophosphorylation of *HER-2* and tyrosine kinase activity[16], which may cause the transformation of cells. In spite of initial reports on the association of this SNP with the risk of a variety of cancers, there is still an ongoing debate on this topic. To our knowledge, this is the first study in a Turkish gastric carcinoma group investigating the existence of the Ile/Val genotype. Although the study group here is small, the 20% occurrence rate in this Turkish gastric carcinoma patient group is comparable to the reported 21% occurrence rate in a Japanese gastric carcinoma group of 212 patients[25]. It suggests a significantly increased risk of gastric cancer development. The association of this genotyping with stage IV gastric cancers also supports this finding.

Overexpression of c-erbB-2, on the other hand, has been associated with poor prognosis and shorter overall survival[14, 30-32] and may be used as a predictive marker for the response to therapy in some cancers[33]. However, a controversy also continues in terms of correlation between the c-erbB-2 protein expression and gastric cancer prognosis. There have been reports correlating the lymph node metastasis and/or liver metastasis with c-erbB-2 expression[14-17], whereas others showed no correlation between the clinicopathologic findings and c-erbB-2 expression[18,19]. Results of this study also correlate with a previous report by Dursun *et al*[34] in terms of positive membranous c-erbB-2 immunoreactivity within a Turkish gastric carcinoma group. Like suggested previously[34] this study has shown association of positive membranous c-erbB-2 immunoreactivity with intestinal type carcinomas ($P = 0.007$), and stage III-IV carcinomas ($P = 0.004$). However, it has to be taken into consideration that both studies have worked with small patient groups leading to a decreased statistical power to detect a positive association of any kind. Thus, further investigation with a desirable study group size is necessary to assess the importance of c-erbB-2 expression in gastric carcinomas for the Turkish population. Nevertheless, results of this study suggest increased c-erbB-2 expression may be considered as a poor prognostic indicator.

In conclusion, this study suggests that the tested SNP in the transmembrane domain-coding region of *HER-2* could be associated with development of gastric carcinoma and may serve as a predictor of risk of malignant phenotype of gastric cancer for the Turkish population. Although the association of the *HER-2* immunoreactivity with clinicopathologic characteristics, especially with intestinal type carcinomas, is also suggested, this has to be confirmed with a larger sample size.

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RAPID COMMUNICATION

Ultrasonic interventional analgesia in pancreatic carcinoma with chemical destruction of celiac ganglion

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Abstract

AIM: To detect the therapeutic effects of chemical destruction of celiac ganglion in patients with pancreatic carcinoma with intractable pain.

METHODS: Ninety-seven cases with advanced pancreatic carcinoma received chemical destruction of celiac ganglion-5 mL pure alcohol injection around celiac artery under ultrasonic guidance. The changes of visual analogue scale (VAS), serum substance P (Sub P), β-endopeptide (β-EP) and T-lymphocyte subtypes level were compared between pre- and post-therapy.

RESULTS: Successful rate of puncture was 98.7%, with one failure. No serious complications such as traumatic pancreatitis, pancreatic fistula, abdominal cavity hemorrhage or peritoneal infection occurred. VAS, serum Sub P and β-EP level significantly changed after treatment (8.0 ± 2.3 *vs* 4.6 ± 2.1, 254.1 ± 96.7 *vs* 182.4 ± 77.6, 3.2 ± 0.8 *vs* 8.8 ± 2.1, $P < 0.01$, $P < 0.05$, $P < 0.01$) with complete relief rate 54.2%, partial relief rate 21.9%, ineffective rate 12.5% and recurrent rate 10.7%. The T-lymphocyte subtypes level remarkably increased when compared with that of pre-therapy (46.7 ± 3.7 *vs* 62.5 ± 5.5, $P < 0.01$).

CONCLUSION: Our study suggests that chemical destruction of celiac ganglion under ultrasonic guidance is highly safe, and can evidently relieve cancer pain and improve the cellular immunity in patients with advanced pancreatic carcinoma.

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Key words: Pancreatic carcinoma; Analgesia; Chemical destruction; Ultrasonic intervention; Celiac ganglion

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INTRODUCTION

Pancreatic carcinoma (PC) is a hyper-malignant disease. Its onset is insidious but its development is aggressive. The radical excision rate is lower than 25%. About 70% of the patients with PC have obvious symptoms of pain and at the late stage all PC patients suffer from severe and intractable pain. The pain has been the chief complaint of the patients with PC, which has significantly decreased their life quality. Thus, to improve the living quality of patients with PC has become the main task for palliative treatment. In 1998, we began to perform chemical destruction of celiac ganglion by ultrasonic intervention in patients with PC in an effort to reduce cancer pain. The results suggested that this method is effective in alleviating the pain caused by PC and can up-regulate the cellular immune level.

MATERIALS AND METHODS

Patients

Our study included 97 patients, 64 males and 33 females, aged between 38 to 79 years, with body weight ranging from 41 to 78 kg (Table 1). Seventy-two were definitely diagnosed as PC by operation (including 26 by pathological biopsy) and 25 by B-ultrasound, CT or MRI (including 10 combined with the increase of CA19-9 value). All the patients suffered from pain in the abdomen and back, characterized by persistently or intermittently attacking pain. According to visual analogue scale (VAS), 29 had moderate pain, 63 had severe pain, and 5 had mild pain. The duration of pain was between 11 to 67 d. All the patients depended on drugs to alleviate the pain before treatment.

Methods

Instruments and reagents used in the study were color B-ultrasonic diagnostic apparatus Aloka-680EX made in Japan, 3.5 MHz convex probe, puncture shelf Aloka MP-2411B made in Japan, puncture needle CNF2220 made in USA, medical analytical anhydrous alcohol and procaine injection (10 g/L). All the patients were fasted for less

Table 1 General data of 97 PC cases

Location of tumor	Diagnosis					Size of tumor (cm)	Metastasis				
	Patients *n*	Operation	Image+ A19-9	Image			Liver	Lung	Lymph node	Colon	Spinal column
Head of pancrreas	55	46	4	5	4.1 ± 2.3	7	-	15	1	-	
Neck of pancrreas	17	13	-	4	5.5 ± 3.8	2	-	3	1	-	
Body of pancrreas	12	5	3	4	4.7 ± 3.1	-	-	3	-	-	
Tail of pancrreas	7	7	-	-	8.6 ± 3.5	-	-	4	-	-	
Whole pancreas	6	1	3	2	-	-	1	2	-	1	

than 12 h before operation; then they were given procaine skin test and VAS training. They were required to catch how to exactly express the degree of pain with VAS and received the compatibility test of posture and respiration. If the patient felt too nervous, he was given intramuscular injection of 10 mg valium 30 min before operation.

Chemical destruction of celiac ganglion

By B-ultrasonic scanning when the patient was lying, the situation around origin plane of celiac arterial trunk was confirmed as the target region. The point of puncture was accordingly confirmed. Routine sterilization was performed on the abdomen and probe. Puncture was performed after local anesthesia of the insert path of needle. B-ultrasound dynamically supervised the course of puncture until the needle arrived at the target region, which was symbolized with heaving impulse and no blood when the syringe was drawn back. Then the patient was given 10 mL procaine injection (10 g/L). If the patient felt significant alleviation of pain or felt warm on his abdomen, the puncture was considered successful. After that, anhydrous alcohol (15 mL) was given to destroy the ganglion. Post-operational fasting, fluid infusion and anti-infectious treatment were performed. If all procedures were carried out smoothly, the patient could then be given normal diet 24 h after operation.

Criteria of curative effects

We used 5 different terms to describe the curative effects: complete relief (CR), partial relief (PR), mild relief (MR), no relief (NP) and recurrence (RC). Those patients who felt no pain and unnecessary to take pain-killers were described as CR. Those who felt significant alleviation of pain after operation but still needed pain-killers occasionally were described as PR. Those who felt a little alleviation after operation but still needed pain-killers because the pain disturbed their sleep were described as MR. Those who felt no change between before and after operation were described as NR. Those who felt alleviation after operation but the pain recurred soon were described as RC.

Visual analogue scale (VAS)

A direct line of 10 cm in length was used. Its both ends expressed 0 and 10, which stood for no pain and the severest pain, respectively. Patients marked the location of pain by themselves, which was used as the basis of quantification.

Sub P and β-EP

Fasting cubital venous blood (4 mL) was averagely grouped: 2 mL Sub P and 2 mL β-EP blood specimens. Iniprol (1000 U) and heparin (10 g/L, 20 U) were added into the Sub P blood specimen. At the temperature of 4 ℃, centrifugation, at a speed of 3000 r/min was performed for 15 min. The upper plasma was preserved at the temperature of -70 ℃.

The β-EP blood specimen was placed in a plastic tube in which there were EDTA-Na2 (6 mg) and bacitracin (160 μg). Then, low temperature centrifugation was performed and the plasma was preserved at the temperature of -20 ℃. The Sub P and β-EP kits were provided by Neurobiological Department of the 2nd Military Medical University and were assayed with the automatic radioimmunoanalyzer FJ-2008P made in Xi'an The unit of Sub P and β-EP was ng/L and nmol/L, respectively.

Assay of T lymphocyte subtype

Fasting venous blood (5 mL) was sampled. Luminescence immunoassay and flow cytometry were used to determine the level of CD_3^+, CD_4^+, and CD_8^+ (cluster of differentiation) and the results were expressed as the percentage of positive cells. MFITC was used to mark the mcAb rats' anti-human CD_3^+, CD_4^+, and CD_8^+ and FITC was used to mark the IgG of rats. All the reagents were purchased from the American Santa Company. Thirty-eight healthy persons were tested as control group.

Statistical analysis

All data were expressed as mean ± SD. *t* test was used to examine the significance of the difference between before and after the treatment and among the groups. *P* value < 0.05 was taken as significant.

RESULTS

The success rate of puncture was 98.7% (96/97). Puncture failed in one patient, who had PC complicated with portal hypertension and there were many variciform veins along the puncture route. In addition, the patient had poor tolerance to puncture posture. We had to give up puncturing on him because the puncture needle could not safely arrive at the target region. In the other 96 patients with successful puncture, there were no such complications as traumatic PC, abdominal cavity hemorrhage, pancreatic fistula or peritoneal infection. Of them, the pain was

Table 2 VAS, plasma Sub P (ng/L) and β-Ep (nmol/L) pre- and post-operation (mean ± SD)

Operation	VAS	Sub P	β-EP
Pre-	8.0 ± 2.3	254.1 ± 96.7	3.2 ± 0.8
Post-			
1 d	5.2 ± 1.8[b]	231.3 ± 101.3	5.1 ± 1.2[f]
3 d	4.6 ± 2.1[b]	182.4 ± 77.6[c]	8.8 ± 2.1[f]
7 d	4.3 ± 1.3[b]	174.5 ± 90.3[c]	9.2 ± 1.8[f]
15 d	3.7 ± 2.0[b]	154.7 ± 81.2[c]	7.6 ± 2.0[f]
30 d	4.1 ± 1.9[b]	188.2 ± 90.6[c]	6.7 ± 1.6[f]

VAS: [a]$P < 0.05$, [b]$P < 0.01$ *vs* Preoperation; Sub P: [c]$P < 0.05$, [d]$P < 0.01$ *vs* Preoperation; β-EP: [e]$P < 0.05$, [f]$P < 0.01$ *vs* Preoperation.

Table 3 Blood T cell subtypes (%) and β-EP (nmol/L) pre- and post-operation (mean ± SD)

Index	Control (n = 38)	Therapeutic (n = 96)			
		Pre-	Postoperation		
			3 d	7 d	15 d	30 d
CD_3^+	70.3 ± 4.9	46.7 ± 3.7	48.8 ± 5.1	58.7 ± 3.7[b]	62.5 ± 5.5[b]	61.2 ± 6.1[b]
CD_4^+	46.6 ± 5.3	31.2 ± 5.0	33.1 ± 3.8	37.6 ± 4.1[d]	40.6 ± 4.4[d]	39.6 ± 3.1[d]
CD_8^+	23.4 ± 4.1	15.0 ± 2.5	14.7 ± 2.8	20.9 ± 3.0[f]	22.0 ± 2.7[f]	21.4 ± 3.2[f]
β-EP	8.7 ± 2.3	3.2 ± 0.8	8.8 ± 2.1[h]	9.2 ± 1.8[h]	7.6 ± 2.0[h]	6.7 ± 1.6[h]

CD_3^+: [a]$P < 0.05$, [b]$P < 0.01$ *vs* Preoperation; CD_4^+: [c]$P < 0.05$, [d]$P < 0.01$ *vs* Preoperation; CD_8^+: [e]$P < 0.05$, [f]$P < 0.01$ *vs* Preoperation; β-EP: [g]$P < 0.05$, [h]$P < 0.01$ *vs* Preoperation.

completely relieved in 52; partially relieved in 21; slightly relieved in 11 and the puncture had no effect in 12. The longest follow-up time was 104 d. The pain recurred in 9 patients on the d 11-51 after puncture, respectively. The recurrence rate was 10.7%. The statistical analysis showed that there were significant changes in VAS, Sub P and β-EP on d 3 after puncture: significant decrease of VAS and Sub P ($P < 0.01$ and $P < 0.05$, respectively) and significant increase of β-EP ($P < 0.01$). By the dynamic follow-up on d 7, 15 and 30, the level of VAS, Sub P and β-EP kept stable, suggesting the stability of our treatment effect (Table 2).

The results showed that the level of CD_3^+, CD_4^+, and CD_8^+ in patients with PC was significantly lower than that in control group ($P < 0.01$). On the 3rd d after chemical destruction of celiac ganglion, the level of subtype T increased slightly compared with that of preoperation but with no significance. On the 7, 15 and 30 d after operation, the level of CD_3^+, CD_4^+, and CD_8^+ increased significantly compared with that of preoperation ($P < 0.01$). The level of β-EP remarkably increased on the 3rd d after operation and there was significant difference compared with before operation ($P < 0.01$). Moreover, the serum β-EP was kept at a high level at 7, 15 and 30 d during follow-up (Table 3).

DISCUSSION

Mechanism of ultrasonic intervention on the pain caused by pancreatic carcinoma

The pain caused by PC mostly comes from the stimulation on the pancreatic sensory nerve fiber, which is conducted through the visceral sympathetic nerve[1-3]. Although the distribution of pancreatic visceral nerves is very complex, they concentrate in the celiac ganglion, project to relevant spinal segments and reach the nerve center of central nervous system, consequently causing pain. In clinic, excision of celiac ganglion or plexus can significantly inhibit the obstinate pancreatogenic pain[1]. Therefore, blocking of the celiac ganglion can theoretically alleviate the pain caused by PC. However, it is difficult by ordinary image technique to show the situation of celiac ganglion. The celiac ganglion is situated on the wall of celiac artery and the anatomical position is fixed. B-ultrasound can clearly show the celiac arterial trunk and its neighboring structure. Therefore, abdominal B-ultrasound can provide indirect guidance for the localization of celiac ganglion. Local injection of anhydrous alcohol, through its demyelinization to destruct the celiac ganglion, can permanently block the transmission passage of the nerves, resulting in alleviation of the pain.

Evaluation of the analgesic effect

As a subjective feeling, pain is affected by various factors. Therefore, in order to objectively reflect the changes of pain after treatment, apart from VAS, Sub P and β-EP were chosen as the evaluation indexes of efficiency. Sub P is an important nerve transmitter of pain. Its concentration in the blood is directly proportional to the injury stimulation[4]. Therefore, Sub P can objectively reflect the degree of pain. β-EP is the main endogenous opiate peptide in human, which has the same analgesic effect as morphine. Millan[5] found that the secretion of β-EP in patients with chronic or carcinoma pain decreased and that the secretion of β-EP increased after the alleviation of pain. In addition, the secretion of β-EP was negatively related to the degree of pain.

The results of our study showed that the overall effective rate of analgesia of chemical destruction of celiac ganglion was 87.5% and the effectual rate was 76.1%. Three days after the treatment, both VAS and Sub P decreased significantly ($P < 0.01$ and $P < 0.05$, respectively) whereas β-EP increased remarkably ($P < 0.01$). Our follow-up of one month showed that the levels of VAS, Sub P and β-EP kept stable. Thus we may conclude that this method is effective against the pain caused by PC. Nerve destruction can decrease the release of the dolorogenic Sub P and up-regulate the level of β-EP, by which the pain is alleviated. However, in 12 patients pain was not significantly alleviated in our study. We suppose that the pain was related to the incomplete obstruction caused by metastatic cancer of colon in 2 cases and hepatic metastasis in 9 and in the remaining one patient, bone destruction of lumbar spine shown by imaging examination, so we suggested that there might be some somatic nerve injured which was associated with the low effectiveness of simple visceral nerve blockade on the pain.

There were no severe complications in the patients. Diarrhea was the common symptom after treatment and the occurrence rate was about 61.5% (59/96). The reason was that after the destruction of celiac ganglion, the inhibitory effect on intestinal wall neuron decreased while its excitability increased, which led to the accentuation

of peristalsis and secretion. Oral activated charcoal was effective on relieving the symptom.

Relationship between analgesia and immune function of T cells

Kelsen *et al*[2] reported that in 77 PC patients, there was significant difference in the survival time between the pain group and no pain group ($P < 0.01$), whether the patient had received operation or not. The survival time of the patients with pain was significantly shorter than those without pain, indicating that the pain caused by PC was closely related to the prognosis. Melzack *et al*[6] observed that the level of Ts, TH and null lymphocyte in patients with pain caused by cancers decreased significantly in 1983. They concluded that pain caused by cancers might induce "dormancy" of T subtypes. The relation between the pain caused by cancers and the immunity of cells has attracted more attention because cellular immunity plays the main role in tumor immunology and it can inhibit the occurrence and progress of cancers. In addition, Ts can identify the neoantigen and TH can kill the carcinogenic cells by cytotoxic reaction. Ma *et al*[7] reported that in 70 patients with tumors at the intermediate or late stage, the level of CD_3^+, CD_4^+ and CD_8^+ in the pain group was significantly lower than that in no pain group ($P < 0.01$) and after the pain was alleviated the level of CD_3^+, CD_4^+ and CD_8^+ increased significantly ($P < 0.05$). Thus it can be concluded that pain caused by tumors can lead to the decrease of human immunity and influence the prognosis and effective control of pain can improve the activity of T subtypes. Morphine is the commonly used pain-killer. It has been proved that perennially taking morphine can lead to addiction and resistance to drugs. Moreover, repeated use of morphine can result in atrophy of pleura and decrease of the expression of CD_3^+, CD_4^+ and CD_8^+[8-10]. Therefore, analgesia should be reasonable in quantity taking into account of its influence on immune function. Our results showed that the expression of T subtypes in patients with pain caused by PC was significantly lower than that in control group, indicating the necessity to protect the immune function. One week after the destruction of celiac ganglion, the expression of CD_3^+, CD_4^+ and CD_8^+ remarkably increased ($P < 0.01$), indicating that analgesia with chemical destruction of celiac ganglion can improve the immune suppression in patients with PC and increase the expression of T subtypes.

Presently it is not clear about the mechanism of pain treatment regulating the immune function. Studies have been focused on the endogenous opiate peptides. On the one hand, β-EP, as an endogenous opiate peptide, has the inhibitory function on pain by the κ and μ receptor pathway; on the other hand, β-EP, as an immune derived product, is the active substance with close relation to the nerve, incretion and immune systems[11]. Maddle *et al*[12] found that β-EP has an important regulatory activity on immunity. Its up-regulation can promote T-lymphocyte multiplication, antibody polymerization and cytotoxic function of NK. Brown *et al*[13] found that there are opiate receptors on the immune cells and β-EP can take part in the regulation of T subtypes and promote their differentiation and growth through opiate receptors. Our study showed that the level of β-EP in pain group was significantly lower than that in control group ($P < 0.01$), which is consistent with the literature. After analgesic therapy, the level of β-EP increased significantly ($P < 0.01$), with a similar trend to that of T subtypes. β-EP level increased significantly ($P < 0.01$) on the 3rd day after treatment, which was ahead of that of CD_3^+, CD_4^+ and CD_8^+. Therefore, we presume that β-EP might take part in the immune regulation of CD_3^+, CD_4^+ and CD_8^+.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

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10th World Congress of the International Society for Diseases of the Esophagus
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www.isde.net

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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EDITORIAL

Role of receptor tyrosine kinases in gastric cancer: New targets for a selective therapy

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Abstract

Receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor family participate in several steps of tumor formation including proliferation and metastatic spread. Several known RTKs are upregulated in gastric cancer being prime targets of a tailored therapy. Only preliminary data exist, however, on the use of the currently clinically available drugs such as trastuzumab, cetuximab, bevacizumab, gefitinib, erlotinib, and imatinib in the setting of gastric cancer. Preclinical data suggest a potential benefit of their use, especially in combination with "conventional" cytostatic therapy. This review summarizes the current knowledge about their use in cancer therapy as well as new approaches and drugs to optimize treatment success.

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Key words: Gastric carcinoma; EGFR; Gefitinib; Trastuzumab; Cetuximab; Imatinib; Erlotinib; Bevacizumab

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INTRODUCTION

Gastric cancer is a common, but difficult to treat disease entity. Apart from potentially curative surgery, chemotherapy as well as radiochemotherapy may be applied, but do not cure the disease[1]. Thus, improvement of gastric cancer therapy will depend on novel therapeutic approaches. Receptor tyrosine kinases (RTKs) are a family of 56 proteins characterized by a transmembrane domain and a tyrosine kinase motif[2]. They function in cell signalling and transmit signals regulating growth, differentiation, adhesion, migration, and apoptosis[3]. Aberrant receptor tyrosine kinase activity was initially described in various epithelial cancers; nowadays, it is well accepted that receptor tyrosine kinases play an important role in almost all types of cancer[4,5]. The mutational activation and/or overexpression of receptor tyrosine kinases transforms cells and often plays a crucial role in the development of cancers.

For this reason, RTKs have become targets of immunological inhibitors, the best known being trastuzumab (Herceptin®, directed against the erb B2 receptor, also known as Her2/Neu oncogene) and cetuximab (Erbitux®, active against the epidermal growth factor receptor [EGFR]). In the case of EGFR, pharmacological inhibitors such as gefitinib (Iresssa®) directed at the kinase domain (EGFR-TKI) of the receptor have been developed; other pharmacological inhibitors of tyrosine kinases include imatinib (Gleevec®) which is licensed for the treatment of leukaemia and gastrointestinal stroma cell tumors (GIST).

As tailored concepts of cancer therapy evolve, RTKs offer prime targets for such an individualized approach to cancer treatment. This review examines the information available on this group of receptors, describes the most relevant subsets that have been found in case of gastric cancer, and summarizes data on the use of their inhibitors in clinical studies. In the end, we present new attempts to optimize the efficacy of already available compounds and promising new drug developments.

Receptor tyrosine kinases

RTKs are membrane bound proteins consisting of a ligand-binding domain at the extracellular surface, a single transmembrane segment, and a cytoplasmic part harboring the protein kinase activity. With the exception of the insulin receptor family of RTKs, all known RTKs form monomers in the cell membrane. Ligand-induced dimerization, resulting in autophosphorylation of their cytoplasmic domains, is the major mode of activation of RTKs.

The known 56 RTKs are divided into 21 families with similar structure and the potential of intrafamilial dimerization; their classification has been reviewed by Robinson *et al*[2]. The best known examples are the epidermal growth factor receptor family (erb B1 to B4), the different vascular endothelial growth factor receptor (VEGFR) subtypes, the

fibroblast growth factor (FGF) receptor family, and the platelet derived growth factor (PDGF) receptor family.

RTKs present in gastric cancer

We have recently analyzed the expression of RTK mRNAs in different human cancers, including 12 samples of gastric cancer[6]. The median of relative gene expression levels of the 56 known RTKs in these cancer specimens are illustrated in Figure 1. Our findings suggest that several RTKs, including those of the EGFR family, the FGFR family, and the different VEGFR subtypes are present in gastric cancer and thus offer potential targets for a selective therapy. In the following, we will summarize findings by others regarding the expression of the RTKs in gastric cancer with emphasis on those for which clinically established modalities of therapeutic intervention exist, namely the EGFR family and VEGF.

EGFR family – EGFR and erb B2

The EGF receptor/ligand system seems to be involved in the regulation of gastric mucosal proliferation and progression of gastric carcinoma. Increased EGFR binding has been observed in gastric carcinomas in comparison to adjacent normal gastric mucosa. Moreover, elevated EGFR levels have been found in gastric carcinoma with worse prognostic factors (T3/4, positive lymph-nodes, G3, diffuse-type). In univariate and multivariate analysis, EGFR levels are an independent indicator of poor prognosis[7,8]. A down-regulation of an inhibitor of EGFR activation, EGFR related protein (ERP) occurs in gastric cancer as well[9]. Changes in the constitutive phosphorylation and activity of tyrosine kinases such as EGFR may contribute to differences in cell adhesion and phenotype of gastric cancer cells[10]. Espinoza *et al* described that gastric adenocarcinomas potentially depend upon the TGFalpha-EGFR autocrine loop for growth and exhibit increased aggressiveness in the presence of aberrant p53[11].

The overexpression of the growth factor receptor oncogene erb B2 in gastric carcinomas was first described by Yonemura *et al* in 1991, who reported a 12% positive rate in 189 gastric cancers[12]. These findings were confirmed by many other studies, which provided further evidence that the highest rate of overexpression was found in patients with advanced disease, and that erb B2 altered expression can be considered as an independent predictor of outcome[13,14]. Ougolkov *et al*[15] found a more frequent overexpression of erb B2 in gastric cancers with concurrent liver metastasis than in those without, and concluded that activated erb B2 may be involved in the process of liver metastasis thus suggesting a role for erb B2 overexpression in identifying gastric cancer patients who are at high risk of developing liver metastasis. In contrast, activating mutations of the erb B2 tyrosine kinase seem to be rare events in gastric cancer[16].

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is expressed in many gastric carcinoma cell lines and may play an important role in cell growth directly. VEGFR is virtually ubiquitous in human tumors and may promote tumor growth and metastasis by participating in both paracrine and autocrine pathways as higher levels of VEGFR have been correlated with more aggressive disease[17]. KDR is the main human receptor responsible for the VEGF activity in both physiological and pathological vascular development, and VEGF-KDR signalling pathway has been validated as a priority target for the development of anti-cancer therapy. A first report on 16 patients with early gastric cancer (pT1-T2, N0) suggested that expression of VEGF may indicate an increased risk of recurrence, making VEGF neutralization an interesting therapeutic target in these subjects[18].

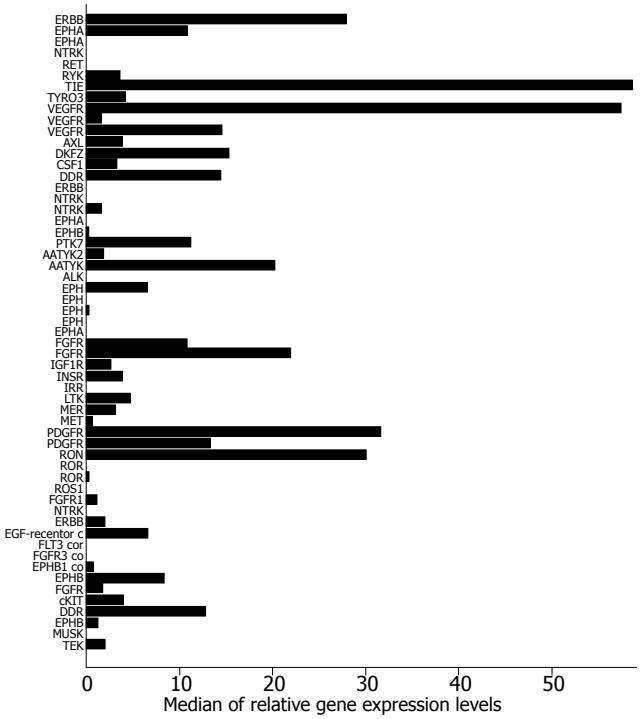


Figure 1 Median gene expression of receptor tyrosine kinases in 12 gastric cancer specimens.

Fibroblast growth factor receptor family

We have previously described an increased expression and autocrine stimulation of fibroblast growth factor (FGF) 2 mRNA in the course of gastric ulcer healing[19]; with respect to gastric cancer, the secretion of growth factors of the FGF family by fibroblasts stimulates proliferation of cancer cells of scirrhous gastric cancer[20]. The expression of FGF2 mRNA (formerly known as basic fibroblast growth factor, bFGF) in gastric cancer specimens is associated with higher microvascular density, tumor progression and a worse prognosis[21]. An orally active inhibitor of FGF-receptor autophosphorylation has been successfully tested in animal models of gastric cancer; further studies are awaited[22].

Platelet derived growth factor receptor

Already more than a decade ago, the expression of platelet derived growth factor (PDGF) and its receptor mRNAs were linked to gastric cancer growth[23]. Later on, the expression of PDGF-A mRNA in biopsy samples of gastric cancer specimens was determined to be a preoperative prognostic marker associated with shorter survival[24]. As

imatinib mesylate as an inhibitor of the PDGFR tyrosine kinase became available, its effectiveness against gastric cancer cells was assessed *in vitro* and *in vivo*. Unfortunately, the agent itself exhibited no cytostatic effect of relevance; yet in combination, imatinib mesylate might serve as an effective chemosensitizer of antitumor drugs, such as 5-FU and paclitaxel for gastric carcinoma, targeting the PDGF/PDGFR-signalling pathway of tumor cells and stromal cells in disease progression and angiogenesis[25].

Leflunomide is a small molecule inhibitor of PDGFR-mediated phosphorylation and inhibits PDGF-mediated cell signalling[26]; it is converted to its main metabolite, SU0020, which interferes with de novo pyrimidine synthesis. At this time, it is not clear whether the mechanism of action of this drug in humans is due to inhibition of PDGF-dependent signalling, inhibition of pyrimidine synthesis, or a combination of both[27-29]. A multi-institutional phase II study in hormone refractory prostate cancer patients with leflunomide found partial responses in 1 of 19 patients, a prostate-specific antigen decline greater than 50% in 3 of 39 patients, and improvement in pain[27].

Hepatocyte growth factor receptor / c-MET

Soman *et al* were able to identify TPR-MET fusion as an event of gastric cancer pathogenesis in certain cases[30]; in addition, c-MET activation seemed to occur[31]. Preliminary data raised in animal models of gastric carcinoma suggest that adenoviral-mediated gene transduction of an HGF antagonist (NK4) results in suppression of tumor growth, invasion, angiogenesis, and metastasis[32,33]. The use of this construct was able to overcome resistance against gefitinib in an animal model of scirrhous gastric cancer[34], yet clinical data is lacking.

USE OF RTK INHIBITORS IN GASTRIC CANCER

Available RTK inhibitors

The inhibitors of tyrosine kinases currently approved by the United States Food and Drug Administration (FDA) for the use in different human malignancies are summarized in Table 1. Substances under development are described in the paragraphs below. If treatment results of RTKIs are to be evaluated, one has to keep in mind that inhibition of a growth factor receptor does not typically aim at killing the cell like "conventional" cytostatics, but stoping proliferation. Therefore, standard assessments of tumor response like complete response, partial response, and stable disease may not reflect the "true" efficacy of such a regime.

Table 1 FDA-licensed tyrosine kinase inhibitors, their targets, modes of action, and clinical use

Agent	Target	Mode of action	Established clinical application
Imatinib mesylate	bcr-abl; PDGFR, c-kit	Kinase inhibitor	CML, GIST, hypereosinophilic syndrome
Gefitinib	erbB1/EGFR	Kinase inhibitor	NSCLC
Erlotinib	erbB1/EGFR	Kinase inhibitor	NSCLC
Cetuximab	erbB1/EGFR	Blocking antibody	Colorectal cancer
Trastuzumab	erbB2/HER	Blocking antibody	Breast cancer
Bevacizumab	VEGF	Neutralizing antibody	Colorectal cancer

Immunological agents

VEGF - Bevacizumab In contrast to the other immunological agents which attach directly to the receptor of the tyrosine kinase, the currently available antibody bevacizumab interrupts the signal cascade by neutralizing the ligand. In a study comprising over 800 patients with metastatic colorectal cancer, the addition of bevacizumab to irinotecan, 5-fluorouracil, and leucovorin as first-line therapy led to a significant increase in survival time[35]. Recent studies concerning the neutralizing antibody bevacizumab, and the small molecule tyrosine kinase inhibitor SU5416, demonstrated that, while unlikely to be effective as monotherapy, incorporation of VEGFR blockade into cytotoxic regimens may increase overall response rates in solid tumors. However, incorporation may also introduce new toxicities, including thromboembolic complications and bleeding[36-38]. Gastrointestinal bleeding, epistaxis and thrombotic events have been seen in 10.3%, 50% and 19.1%, respectively, of the patients receiving bevacizumab alone[39]; these side effects should be taken into consideration when deciding about bevacizumab therapy. There is a phase II trial of bevacizumab, irinotecan, and cisplatin in metastatic or unresectable gastric cancer (National Cancer Institute [NCI] protocol 6447) currently active, of which only the high rate of thromboembolic events in six (25%) of 24 patients (95% CI, 11% to 45%) has recently been reported[40]. However, the incidence of thromboembolic events was not significantly different when compared to a similar irinotecan-based protocol not containing bevacizumab, and there was no difference in the pattern of venous thromboses.

A small molecule inhibitor of VEGFR2 (Vatalanib; PTK787/ZK 222584) has been developed; preliminary data on its use in colorectal cancer exist[41]. With respect to gastric cancer, a Japanese group reported the effects of another VEGFR tyrosine kinase inhibitor (SU6668) in a mouse model of gastric cancer. SU6668 did not directly alter the growth rate of the cancer cells, but inhibited tumor angiogenesis, resulting in the inhibition of tumor dissemination in the peritoneum[42].

Erb B2 / HER-neu – Trastuzumab When considering how EGFR/erb B-targeted therapeutics function, it is important to mention that, in contrast to the small molecule kinase inhibitors, antibodies targeting EGFR and erb B2 have the inherent ability to recruit immune effector cells such as macrophages and monocytes to the tumor through binding of the antibody constant Fc domain to specific receptors on these cells. In xenograft models at least, this mechanism is relevant with the anti-tumor activity of erb B2-targeted trastuzumab[43]. Whether this mechanism has a role in clinical efficacy in cancer patients remains unproven[44]. Trastuzumab (Herceptin) is a humanized monoclonal antibody, which was approved by the United States FDA in 1998 for the treatment of advanced breast cancer. This was the first approval of a monoclonal antibody for use in solid tumor therapy[45]. Recently, it was

investigated whether trastuzumab could affect the growth of HER/neu-overexpressing gastric cancer cells based on the antibody-dependent cell-mediated cytotoxicity (ADCC). It has been demonstrated that these cells could be killed by trastuzumab-mediated ADCC and this correlated with the degree of HER-2/neu expression[46]. However, the trastuzumab-mediated ADCC was significantly impaired when tested in peripheral blood mononuclear cells from patients with advanced disease as compared to those with early disease. Moreover, natural killer cells purified from patients with advanced disease showed less trastuzumab-mediated ADCC in comparison to those from healthy donors. Consequently it has also been postulated that some treatment modalities, such as those involving the use of interleukin 2, could contribute to reverse NK dysfunction, which may be necessary for successful trastuzumab treatment of gastric cancer. Funato *et al*[47] used MKN-7 and KATO-III gastric cancer cells, which express the erb B2 oncogene, to study the mechanism of resistance to cisplatin. They found that erb B2 expression in gastric cancer is related to cisplatin sensitivity, and that anti-erb B2 antisense oligonucleotides could induce increased sensitivity to this drug. Experimental therapy utilizing a different anti-HER mouse-human chimeric monoclonal antibody named CH401 documented efficacy in an *in vivo* model of gastric cancer, yet clinical data are lacking[45].

Epidermal growth factor receptor (EGFR) – Cetuximab

Cetuximab has been approved by the FDA for use in colorectal cancer; cetuximab and other molecules inhibiting the EGFR pathway in colorectal cancer have been recently reviewed[48,49]. One large trial of cetuximab in colorectal cancer comprising 329 patients with irinotecan-refractory metastatic cancer indicated a delayed median time to progression by combining cetuximab with irinotecan[50]. A known adverse characteristic of anti-EGFR therapy is an acne-like skin rash associated with treatment response, which was observed in about three out of four patients[51]. In contrast to gefitinib, the response of cancer cells to cetuximab occurs independent of the mutational status of the EGFR[52,53]. Unfortunately, there are no relevant clinical data available on the use of cetuximab in patients suffering from gastric cancer. As cetuximab is a chimeric antibody which may cause immunological reactions, humanized anti-EGFR antibodies have been developed, one being matuzumab, which currently undergoes phase II trials including studies in patients suffering from gastric cancer[54].

Pharmacological agents – small molecule tyrosine kinase inhibitors

Imatinib: Imatinib is an established and licensed treatment modality in gastrointestinal stroma cell tumors (GIST)[55], but with respect to gastric cancer, only limited data about its use exist. A single study in an animal model of gastric cancer suggested no independent activity of imatinib, but proved an effective chemo- sensitization of antitumor drugs, such as 5-FU and paclitaxel for gastric carcinoma, targeting the PDGF/PDGFR-signalling pathway of tumor cells and stromal cells in disease progression and angiogenesis[25].

EGFR-inhibitors–Erlotinib and Gefitinib

The first representative of this drug class to be approved for cancer therapy was gefitinib (Iressa®) in the third line treatment of non-small-cell lung cancer[56]. A subsequent phase III trial (Iressa Survival Evaluation in Lung cancer, ISEL) failed to demonstrate survival advantage for those patients when compared with placebo[57]. Furthermore, in other malignancies such as gastric carcinoma, preliminary data indicate that treatment efficacy with this regimen is limited as well[58]. A phase II trial to investigate the efficacy, tolerability and pharmacokinetics of gefitinib in pretreated patients with metastatic gastric carcinoma included 75 subjects who were randomised to receive 250 or 500 mg/d gefitinib orally. The authors found that gefitinib monotherapy was generally well tolerated in pretreated patients with gastric metastatic adenocarcinoma, with disease control achieved in 18.3% of cases analyzed. The most common drug-related adverse events were diarrhea, rash and anorexia. The only dose-related adverse events were rash (25.0% at 250 mg/d *vs* 44.7% at 500 mg/d) and anorexia (8.3% at 250 mg/d *vs* 15.8% at 500 mg/d). Rojo *et al*[59]evaluated immunohistochemically the percentage of tumor cells expressing EGFR, pEGFR (the activated phosphorylated form), pMAPK, pAkt (phosphorylated Ser473) and Ki67, before and after treatment with gefinitib. Prior to treatment EGFR expression was found in 62.5% of tumors, whereas pEGFR levels were significantly reduced after the treatment. However, a decreased proliferation was observed only in those tumors with low levels of pAkt, suggesting a role for the PI3k-Akt pathway in gefinitib resistance. Recent studies suggested that clinical response to gefitinib in lung cancer depends on the presence of somatic mutations of the EGF receptor in the tumor which enhance the responsiveness of the receptor to EGF ligand and increase its sensitivity to inhibition by gefitinib[60-62]. In the case of gastric cancer, no such mutations are known. Data on the use of erlotinib (Tarceva®; OSI-744) in gastric cancer is limited to a single study of 70 patients having either gastric cancer (n = 26) or gastroesophageal junction cancer (GEJC) (n = 44). No patient in the gastric cancer cohort presented an objective response, but five patients in the GEJC cohort did so, one being a complete response. An overall response rate was 11%[63]. The best known therapeutics targeting members of the EGFR family which are currently available or under investigation are summarized in Table 2.

Attempts to optimize treatment efficacy of RTKIs

As resistance against single agents may arise and tumor survival may rely on more than one growth factor pathway, several attempts have been made to optimize treatment efficacy, most of them still being in preclinical testing. The following options exist: (1) Combination of inhibitors of the same pathway (e.g. gefitinib and cetuximab) in order to further enhance signal abrogation; (2) To combine inhibitors of different RTK pathways or apply non-selective inhibitors of several pathways in case the cancer cell loses its reliance on one specific pathway; (3) To select cells dependent on growth factor stimulation by use of other cytotoxic drugs or radiation and subsequently eliminate these by an immunological or pharmacological

RTKI.

Enhanced abrogation of one RTK pathway by combining different inhibitors

In 2004, two groups described the use of cetuximab combined with gefitinib and erlotinib as enhanced abrogation of the EGFR signal cascade in *in vitro* as well as *in vivo* models. Matar *et al*[64] utilized an EGFR-dependent human tumor xenograft model and found a synergistic effect on cell proliferation and superior inhibition of EGFR-dependent signalling and induction of apoptosis. Even suboptimal doses of gefitinib and cetuximab given together resulted in a complete and permanent regression of large tumors. In the combination-treated tumors, there was a superior inhibition of EGFR, mitogen-activated protein kinase, and Akt phosphorylation, as well as greater inhibition of cell proliferation and vascularization and enhanced apoptosis. Using cDNA arrays, 59 genes could be identified that were coregulated and 45 genes differentially regulated, including genes related to cell proliferation and differentiation, transcription, DNA synthesis and repair, angiogenesis, signalling molecules, cytoskeleton organization, and tumor invasion and metastasis. Huang *et al*[65] reported similar findings in head and neck tumors and in a model of lung cancer; they observed that gefitinib and erlotinib retained the capacity to inhibit tumor cell growth in case of cetuximab resistance. Again, the combination of antibody and kinase inhibitor resulted in more profound tumor regression and regrowth delay.

Another group applied trastuzumab and an inhibitor of EGFR and erb B2 tyrosine kinases, lapatinib, in erb B2-overexpressing breast cancer cell lines. Only in combination, treatment resulted in a markedly downregulated survivin protein expression and enhanced tumor cell apoptosis thus suggesting a potential improvement in clinical response[66]. But not all findings support the idea of combining inhibitors of the same pathway: in an *in vitro* model using two human epidermoid cell lines the combination of cetuximab and gefitinib demonstrated antagonistic effects. Administration of either drug alone led to a diminution in EGFR levels, while their combination increased the cellular expression of EGFR. These findings suggest that new and tempting treatment strategies on the EGFR target consisting in a double hit with a monoclonal antibody and a TKI must be considered with caution[67]. Data with regard to gastric cancer are currently lacking.

Inhibition of several RTK pathways: combination of different substances, non-selective inhibitors

Cancers possessing complex kinase profiles may respond better to a multimodal therapy tackling several pathways; and such regime may simultaneously reduce the emergence of resistance. Therefore a combination of different agents or a single "unspecific" inhibitor of several pathways may offer advantages over inhibition of a single pathway.

Combinations of different agents: As there is currently no single known inhibitor of all relevant receptor tyrosine kinases, combination of the well evaluated agents offers a straightforward concept of therapy, taking into account that in most cancer therapies different substances are being combined. First data were raised in phase I or II breast cancer trials by combining trastuzumab with small molecule inhibitors of EGFR such as gefitinib or erlotinib indicating that this combination provided a well tolerated targeted therapy with preliminary evidence of antitumor activity[68,69]. As activation of the EGF receptor may induce vessel formation by cellular liberation of VEGF, approaches blocking both pathways possess certain attractiveness. In conditions of limiting VEGF, EGF plays an important role in endothelial cell proliferation, survival, and sprouting of small vessels. Animal data suggest that combined inhibition of EGFR and VEGFR pathways may produce synergistic results and that resistance to EGFR inhibition may be overcome by inhibition of VEGFR tyrosine kinase[70,71]. In an *in vivo* model, the combination of anti-VEGF-R and anti-EGF-R therapies was effective in inhibiting gastric cancer growth whereas the decrease in tumor growth in mice treated with DC101 (an anti-VEGF-R antibody) or cetuximab alone did not reach statistical significance[72]. As mentioned above, the addition of an inhibitor of the hepatocyte growth factor was able to overcome resistance against gefitinib resulting from the interaction of stromal and cancer cells in an animal model of scirrhous gastric cancer[34].

Inhibitors not selective for a single pathway: Agents targeting multiple RTKs are currently undergoing preclinical testing or phase I studies. These include AEE788 (directed against EGFR, erbB2, VEGFR2; Novartis), BAY 43-9006 (Sorafenib; Raf kinase, VEGFR2, PDGF-R beta; Bayer), SU11248 (PDGF-R, VEGFR, c-kit-R, FLT3-R; SUGEN), and ZD6474 (VEGFR and EGFR, AstraZeneca), all designed to inhibit multiple mechanisms of tumor growth in addition to conventional chemotherapy[73-76]. Similar to the combination of anti-EGFR and anti-VEGFR antibodies mentioned above, McCarthy *et al*[77] tested the inhibitor of both tyrosine kinases, ZD6474, in an orthotopic model of gastric cancer. The agent led to marked

Table 2 Therapeutics targeting the EGFR family

Agent	Type	Target	Status
Trastuzumab	Humanized mAb	erbB2	Approved for breast cancer
Pertuzumab	Humanized mAb	erbB2	Phase II trials
Cetuximab	Chimeric mAb	EGFR	Approved for colorectal cancer
Matuzumab	Humanized mAb	EGFR	Phase II trials
Panitumab	Humanized mAb	EGFR	Trials ongoing
Gefitinib	TKI	EGFR	Approved for NSCLC
Erlotinib	TKI	EGFR	Approved for NSCLC
Lapatinib	TKI	EGFR/erbB2	Phase III trial / breast cancer
AEE788	TKI	EGFR/ erbB2/ VEGFR	Phase I trials
CI-1033	Irreversible TKI	EGFR/erbB2	Phase II trials
EKB-569	Irreversible TKI	EGFR/erbB2	Phase II trials
EXEL 7647 /TKI EXEL 0999		EGFR/ erbB2/ VEGFR	Phase I trials

(incomplete list; modified according to ref. 44)

inhibition of tumor growth, tumor cell proliferation, and decrease in microvessel density. The authors concluded that therapies such as ZD6474 that target two distinct aspects of tumor growth, angiogenesis and tumor cell proliferation, warrant further investigation.

Combination of standard chemotherapeutic agents and radiation with inhibition of RTKs

"Conventional" chemotherapy and RTKIs: The combination of antibody based therapies with conventional therapy has become a standard procedure in many cancers. For example, in colorectal cancer, cetuximab or bevacizumab is typically combined with irinotecan-based regimes[35,50]. One of the first reports on the use of an RTKI, trastuzumab, in breast cancer documented the inhibition of DNA repair subsequent to DNA damage by cisplatin by an antibody to Her2/Neu. Therapy with this antibody led to a 35%-40% reduction in repair of cisplatin-DNA adducts after cisplatin exposure and, as a result, promoted drug-induced killing in target cells[78]. Another study using human tumor xenografts found that gefitinib caused growth inhibition of tumors and enhancement of the activity of a number of cytotoxic drugs, but neither was dependent on high levels of EGFR expression[79]. In an animal model of pancreatic cancer, inhibiting phosphorylation of EGFR, VEGFR, and PDGFR by appropriate RTKIs in combination with gemcitabine enhanced the efficacy of gemcitabine alone, resulting in inhibition of experimental human pancreatic cancer growth and significant prolongation of survival[80]. Similar results were obtained in a model of estrogen receptor-positive breast cancer in which successful cooperation of the dual erb B1/B2 inhibitor lapatinib with tamoxifen was evidenced[81]. Yet there are pitfalls of combinatorial therapy: the combination of two substances may not always be beneficial; for example, the combination of tamoxifen and trastuzumab in estrogen-receptor positive breast cancer may be less effective than either substance alone possibly due to an increase in erb B2 signalling pathways that occurs when tamoxifen is added to trastuzumab[82]. In case of gefitinib, addition of this RTKI to platin-based chemotherapy in non-small cell lung cancer (INTACT 2 trial) showed no added benefit in survival, TTP, or RR compared with standard chemotherapy alone. This large, placebo-controlled trial confirmed the favorable gefitinib safety profile observed in phase I and II monotherapy trials[83]. Currently only data from animal or *in vitro* testing exist with respect to gastric cancer; Park *et al*[84] investigated the effect of gefitinib combined with oxaliplatin, 5-fluorouracil, or paclitaxel in a gastric cancer cell line, SNU-1. This study demonstrated the antitumor activity and a significant cell cycle arresting effect induced by gefitinib in SNU-1 human gastric carcinoma cells, and its synergistic interaction with oxaliplatin and paclitaxel. There are preliminary data on a multicenter phase II study of irinotecan, cisplatin, and bevacizumab in gastric or gastroesophageal adenocarcinoma which indicate an excellent disease control rate of 13/15 cases; in a subset of ten patients with measurable disease who had received at least two cycles of therapy, 5 partial responses, 4 minor responses (15%-29% reduction) and 1 stable disease were observed[85]. Other, less toxic substances have been assessed as well. Thus it has previously been documented that gastric tissue exposed to acetylsalicylic acid (ASA) expresses high levels of EGFR[86,87]. A positive loop regulation between COX and erbB2[88] as well as EGFR[89] has been postulated. Since nonsteroidal anti-inflammatory drugs (NSAIDs) might be a tool of carcinoma prevention in the gastrointestinal tract[90] we investigated the mechanisms of a potential synergism of simultaneous cyclooxygenase (COX)- and EGFR-inhibition. It has previously been described that simultaneous administration of COX- and EGFR-inhibitors exerts tumor preventive effects in nude mice[91]. A combination of these two substances with a protein kinase A (PKA) antisense oligonucleotide was able to eliminate tumors in more than half of the animals treated[92]. Preliminary data from our group indicate that acetylsalicylic acid may modulate the expression and activation of EGFR in gastric cancer cells rendering them more susceptible to gefitinib treatment[93].

Radiation therapy and RTKIs: Radiation causes cell death by induction of cellular injury which may rely on subsequent growth factor/receptor tyrosine kinase activation for repair, the combination of radiation and RTKIs seems to be obvious. Currently, however, only data with respect to experimental therapy, especially in animal models, exist. E.g., She *et al*[94] examined the effect of addition of gefitinib to radiation therapy in a nude mouse model of different cancers, including lung and breast cancer. Gefitinib significantly enhanced the antitumor action of radiation therapy against the test tumors without significant adverse effects, increasing the therapeutic selectivity of ionizing radiation in these model systems. In a similar model, PTK787/ZK222584, a specific inhibitor of VEGFR tyrosine kinases, was tested. Tumors vascularized by radiation-damaged vessels responded to PTK787/ZK222584 with longer latency and slower growth rate than controls, and a trend toward further increase in necrosis, indicating that irradiated tumor vessels are more susceptible to VEGFR inhibition than unirradiated vessels[95].

CONCLUSION

Receptor tyrosine kinases participate in several steps of tumor formation including proliferation and metastasis formation. As several of them are upregulated in gastric cancer, they offer potential prime targets for a tailored therapy. Unfortunately, only preliminary data exist on the use of the currently clinically available drugs such as trastuzumab, cetuximab, bevacizumab, gefitinib, erlotinib, and imatinib in the setting of gastric cancer. However, phase II trials are underway to examine the potential of these drugs in adenocarcinoma of the stomach. As RTK inhibitors with a broad range are being developed, the potential usefulness of this drug class is most likely to further increase as preclinical data in models of gastric cancer already indicate their effectiveness. The approach of combining RTKIs with "conventional" means of tumor therapy such as cytostatics and radiation therapy is most likely to find its way into clinical application in near future. RTKIs alone typically only inhibit tumor growth and do not aim at killing the cancer cell, but they might prove to

be an effective chemosensitizer of other antitumor drugs as they are able to block the process of cellular restitution following injury caused by radiation or "conventional" cytostatics. Different trial designs may be necessary given that some RTKIs might only work in small subsets of patients (for example, the fate of gefitinib in non-small cell lung cancer) and often improve patients' condition without leading to an objective response. Genomic or proteomic analysis of aberrations in individual patients may be necessary to decide about a tailored therapy.

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REVIEW

Antibiotics and probiotics in treatment of inflammatory bowel disease

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Abstract

Many experimental and clinical observations suggest that intestinal microflora plays a potential role in the pathogenesis of inflammatory bowel disease (IBD). Manipulation of the luminal content using antibiotics or probiotics represents a potentially effective therapeutic option. The available studies do not support the use of antibiotics in ulcerative colitis (UC). Antibiotics are effective in treating septic complications of Crohn's disease (CD) but their use as a primary therapy is more controversial, although this approach is frequently and successfully adopted in clinical practice.

There is evidence that probiotic therapy may be effective in the prevention and treatment of mild to moderate UC. In contrast, a lack of successful study data at present precludes the widespread use of probiotics in the treatment of CD.

Both antibiotics and probiotics appear to play a beneficial role in the treatment and prevention of pouchitis and further trials are warranted to fully quantify their clinical efficacy.

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Key words: Inflammatory bowel disease; Intestinal microflora; Antibiotics; Probiotics

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INTRODUCTION

The rationale for using antibiotics and probiotics in the treatment of inflammatory bowel disease (IBD) is based on convincing evidence implicating intestinal bacteria in the pathogenesis of the disease[1].

The distal ileum and the colon are the areas with the highest bacterial concentrations and represent the sites of inflammation in IBD. In addition, pouchitis, the non-specific inflammation of the ileal reservoir after ileo-anal anastomosis, appears to be associated with bacterial overgrowth and dysbiosis. Furthermore, pouchitis does not occur prior to closure of the ileostomy.

Patients with Crohn's disease (CD) consistently respond to diversion of faecal stream, with immediate recurrence of inflammation after restoration of intestinal continuity or infusion of luminal content into the bypassed ileum[2,3]. Moreover, the composition of the enteric flora is altered in patients with IBD, and enteric bacteria or their products have been found within the inflamed mucosa of patients with CD[4]. Increased number of aggressive bacteria such as *Bacteroides,* adherent/invasive *Escherichia coli* and enterococci, and decreased number of protective lactobacilli and bifidobacteria have been observed in IBD[5].

However, the most compelling evidence that intestinal bacteria play a role in IBD has been derived from animal models. Although there is a great diversity in genetic defects and immunopathology, a consistent feature of many transgenic and knockout mutant murine models of colitis is that the presence of normal enteric flora is required for full expression of inflammation[6]. Indeed, there is evidence that immunological tolerance to commensal bacteria is lost in patients with IBD[7,8]. These findings have led to the proposal that manipulation of intestinal microbiota flora, either with antibiotics or probiotics, may be therapeutic in IBD. Some suggested mechanisms of action of antibiotics and probiotics are shown in Table 1.

There is a growing body of evidence from animal studies and clinical trials that antibiotics and probiotics have therapeutic effects in ulcerative colitis (UC), CD and pouchitis.

ANTIBIOTICS

Animal model studies

In several rodent models the use of broad-spectrum an-

Table 1 Suggested mechanisms of action of antibiotics and probiotics

Antibiotics	Probiotics
Eradication of bacterial antigenic triggers	Inhibition of pathogenic enteric bacteria by:
Elimination of bacterial overgrowth	decreasing luminal pH
Reduction of pro-inflammatory bacterial toxins	secretion of bacteriocidal proteins
Potential immunosuppressive properties of antibiotics	resisting colonization
	blocking epithelial binding
	Improvement in epithelial and mucosal barrier function by:
	production of short-chain fatty acids
	enhancing mucus production
	increasing barrier integrity
	Alteration of immunoregulation by:
	increasing interleukin-10 and TGFβ, and decreasing TNF levels
	increasing IgA production

tibiotics can both prevent and treat experimental colitis, whereas metronidazole and ciprofloxacin can only prevent experimental colitis but cannot reverse the established disease[9-13]. Broad-spectrum antibiotics are effective in almost all models of acute and chronic colitis[13-16], but they have only a transient efficacy in HLA-B27 transgenic rats[17]. Interestingly, ciprofloxacin and metronidazole have selective efficacy in different colonic regions of interleukin-10 (IL-10) knockout mice, suggesting that different bacteria cause inflammation in different colonic segments[15]. These studies suggest that most clinical forms of IBD may respond to a specific combination of broad-spectrum antibiotics.

Ulcerative colitis

Only a few trials on the use of antibacterial agents have been carried out in UC and the results are controversial. Most clinicians have used antibiotics as an adjuvant therapy for severe UC. Dickinson *et al*[18] carried out a double-blind controlled trial on the use of oral vancomycin as an adjunct for acute exacerbations of idiopathic colitis and found that there is no significant difference between the two treatment groups, with only a trend towards a reduction in the need for surgery in patients treated with vancomycin[18].

Intravenous metronidazole used in conjunction with corticosteroids, is as effective as placebo in inducing remission in patients with severe UC[19]. In a double-blind, placebo-controlled trial in patients with acute relapse of UC, Burke *et al*[20] randomized 84 patients to receive corticosteroids plus oral tobramycin or placebo and found that after 1 wk of treatment, 74% of patients in the tobramycin treatment group and 43% in the placebo group ($P < 0.003$) achieve complete symptomatic remission. However, the combination of tobramycin and metronidazole does not have any beneficial effect when compared with a standard steroid treatment in severely acute UC[21].

Mantzaris *et al*[22] investigated ciprofloxacin in a randomised, placebo-controlled study and randomized 70 patients with mild to moderate active UC to receive either 250 mg ciprofloxacin twice a day or placebo for 14 d and found that 70.5% of patients in the ciprofloxacin group and 72% in the placebo group achieve remission. Moreover, a short course of intravenous ciprofloxacin is not effective as an adjunctive treatment to corticosteroids in severe UC[23]. In contrast, some efficacy of ciprofloxan has been observed in a more recent randomised placebo-controlled trial when ciprofloxacin is administered for 6 mo to patients with active UC poorly responding to conventional therapy with steroids and mesalazine[24]. At the end of the study, the treatment-failure rate was 21% in the ciprofloxacin-treated group and 44% in the placebo group ($P < 0.002$). This difference was detected using clinical criteria; while endoscopic and histological findings showed differences only at 3 mo but not at 6 mo.

The non-absorbable broad-spectrum antibiotic, rifaximin, was investigated in a small controlled study to evaluate its efficacy and systemic absorption in patients with moderate to severe active UC refractory to steroid treatment. Twenty-eight patients were randomised to receive either rifaximin 400 mg twice daily or placebo for 10 d as an adjunct to standard steroid treatment. Although there is no significant difference in the clinical efficacy score between the two treatments, only rifaximin determines a significant improvement in stool frequency, rectal bleeding and sigmoidoscopic score[25].

Whilst rifaximin does not permanently alter the colonic microbiota, resistant *Bifidobacterium* species have been found after 3 intermittent courses in patients with UC[26].

Crohn's disease

Broad-spectrum antibiotics are widely used to treat CD[27], but large controlled trials have not yet been performed (Table 2).

Metronidazole has been the most investigated agent. In 1978, Blichfeldt *et al*[28] found that there is no difference between metronidazole and placebo-treated patients in a placebo-controlled, double-blind, crossover trial. However, a positive trend in favour of metronidazole is observed when only the colon is involved[28]. In the National Cooperative Swedish study, metronidazole has been compared with sulphasalazine as a primary treatment for Crohn's disease. Although no significant difference is found between the two groups, metronidazole is effective in patients who fail to respond to sulphasalazine[29]. In

Table 2 Antibiotics trials in active Crohn's disease

Study	Patients (*n*)	wk	Main outcome	Study design	Treatment schedules
Blichfeldt *et al*[28]	22	8	Improvement (clinical/lab score)	DB crossover study	MZ (+ SASP/CS) Placebo (+ SASP/CS)
Ursing *et al*[29]	22	16	Change in CDAI and orosomucoid	DB crossover study	MZ SASP
Ambrose *et al*[30]	72	4	Improvement (Clinical/lab.score)	DB RCT	MZ CO, MZ/CO, placebo
Sutherland *et al*[31]	99	16	Change in CDAI from baseline	DR RCT	MZ (10/20 mg/kg) Placebo
Prantera *et al*[32]	41	12	Clinical remission (CDAI < 150)	NB RCT	MZ + Cipro Steroids
Colombel *et al*[34]	40	6	Clinical remission (CDAI < 150)	NB RCT	CIPRO 5-ASA
Arnold *et al*[35]	47	24	Change in CDAI	NB RCT	CIPRO (+ conc drugs) Placebo (+ conc drugs)
Steinhart *et al*[33]	134	8	Clinical remission (CDAI < 150)	DB RCT	MZ+CIPRO (+ bud 9 mg) Placebo (+ bud. 9 mg)

CDAI: Crohn's disease activity index.

another study, metronidazole was used either as a single therapy or in combination with cotrimoxazole and compared to cotrimoxazole alone and a double placebo in patients with a symptomatic relapse of CD, which shows that after four weeks of treatment there is no difference in response among the three treatment groups[30]. In a Canadian randomised, placebo-controlled trial, Sutherland *et al*[31] demonstrated that treatment with metronidazole for 16 wk significantly decreases the Crohn's disease activity index (CDAI), but no difference is found in the rates of remission compared with placebo. As in the Swedish study[29], the Canadian study found that metronidazole is effective for colonic and ileocolonic CD, but not for ileitis. Unfortunately, metronidazole has numerous side-effects including nausea, anorexia, dysgeusia, dyspepsia and peripheral neuropathy, which limit its use in approximately 20% of patients.

An antibiotic combination was used in an Italian randomised controlled study[32] in which 250 mg metronidazole four times daily plus 500 mg ciprofloxacin twice daily were compared to a standard steroid treatment for 12 wk. No significant differences were reported in the rates of remission between treatments (46% with ciprofloxacin plus metronidazole and 63% with methylprednisolone), suggesting that this antibiotic combination is a potential alternative to steroid treatment in the acute phase of CD[32]. In another trial[33], this combination of metronidazole and ciprofloxacin was supplemented with budesonide (9 mg/d) for active CD. No difference was registered compared to placebo, but the overall response in the two groups was lower than that in previous studies using budesonide, suggesting that antibiotic treatment is more effective in colonic disease than in isolated small bowel disease.

Ciprofloxacin (1 g/d) was compared to mesalazine (4 g/d) in a controlled study[34] of mild to moderate active CD for 6 wk. The results suggest that ciprofloxacin is as efficacious as mesalazine (remission observed in 56% and 55% of patients treated with ciprofloxacin and mesalazine respectively), thus offering a potential alternative treatment for active CD. Furthermore, ciprofloxacin has been shown to be effective when used in combination with standard treatment in patients with resistant disease [35].

Other antibiotics have also been investigated. Shafran *et al*[36] carried out an open-label study on the efficacy and safety of rifaximin (600 mg/d) for 16 wk in the treatment of mild to moderate active CD, and found that at the end of the study, 59% of patients are in remission (CDAI < 150) with a significant reduction of the mean CDAI score compared to baseline ($P < 0.0001$). Leiper *et al*[37] reported that 64% patients have an impressive positive response to clarithromycin, many of whom are unresponsive to other treatments.

Many studies have tried to evaluate the efficacy of antimycobacterial drugs in patients with CD, pursuing the possibility that a strain of *Mycobacterium* might be an aetiological agent in CD. Borgaonkar *et al*[38] performed a meta-analysis of all randomised controlled trials in which antimycobacterial therapy was compared with placebo and found that antimycobacterial therapy is only efficacious in the maintenance of remission after a combined treatment of corticosteroids and antimycobacterial agents. However, the investigator emphasised that because of the high incidence of side-effects and the small number of studies included in the meta-analysis, the data are inconclusive and should be interpreted with caution.

The same antibiotics used to treat luminal CD have been reported to be beneficial for the treatment of perianal CD, but no controlled trials are available[39]. Metronidazole (20 mg/kg) can close 62%-83% fistulae[40,41]. The combination of metronidazole and ciprofloxacin results in an improvement in 64% of patients with closure of fistulae in 21%[42]. Unfortunately, fistulae tend to recur in most patients following cessation of treatment. Although the results of these uncontrolled studies are inconclusive, metronidazole and ciprofloxacin alone or in combination, are used by most clinicians as first-line treatments for patients with perianal disease in conjunction with surgical drainage of abscesses.

The use of antibiotics in the prevention of post-

operative disease recurrence has also been investigated. Rutgeerts *et al*[43] have assessed the efficacy of metronidazole at 20 mg/kg per day in a placebo-controlled double-blind study. In their study, sixty patients were randomised to receive either metronidazole or placebo for 12 wk and endoscopic relapse was evaluated by Rutgeerts score at the end of the treatment. They found that metronidazole significantly decreases the incidence of severe endoscopic relapse (grade 3 or 4) and the clinical recurrence rate. More recently, ornidazole used continuously for 1 year, has been shown to be significantly more effective than placebo in the prevention of clinical and endoscopic recurrence in the neoterminal ileum[44].

Pouchitis

The awareness of the crucial importance that faecal stasis and bacterial overgrowth may play a role in the pathogenesis of acute pouchitis has led clinicians to treat patients with antibiotics.

Antibiotics have become the mainstay of treatment for pouchitis, although controlled trials are not available. Metronidazole is the first-line treatment, and most patients with acute pouchitis respond quickly to its administration of 1-1.5 g/d[45,46]. A double-blind, randomised, placebo-controlled, crossover trial was carried out by Madden *et al*[47] to assess the efficacy of 400 mg of metronidazole three times daily *per os* for two weeks in 13 patients (11 completed both arms of the study) with chronic, unremitting pouchitis. The found that metronidazole is significantly more effective than placebo in reducing stool frequency (73% and 9%), even without improvement in endoscopic appearance and histological grade of activity. However, a significant proportion of patients (55%) may experience side-effects while using metronidazole, including nausea, vomiting, abdominal discomfort, headache, skin rash and metallic taste. Recently Shen *et al*[48] compared the efficacy and side-effects of ciprofloxacin and metronidazole in treating acute pouchitis in a randomised clinical trial. Seven patients received ciprofloxacin (1 g/d) and nine patients received metronidazole (20 mg/kg per day) for 2 wk. The results of this study have shown that both ciprofloxacin and metronidazole are efficacious in the treatment of acute pouchitis. Both reduce the total pouchitis disease activity index (PDAI) scores and lead to a significant improvement in symptoms as well as endoscopic and histological scores. However, ciprofloxacin leads to a greater reduction in PDAI scores as well as improvement in symptoms and endoscopic scores. Furthermore ciprofloxacin is better tolerated than metronidazole (33% of metronidazole-treated patients reported adverse effects, compared with none in the ciprofloxacin group).

Given the management difficulties posed by chronic refractory pouchitis, the use of combined antibiotic treatment has been explored. In an open trial, 18 patients with active pouchitis not responding to standard therapy (metronidazole or ciprofloxacin) for 4 wk, were treated orally with rifaximin (2 g/d) plus ciprofloxacin (1 g/d) for 15 d. Symptom assessment, endoscopic and histological evaluations were performed at screening and after 15 d using PDAI scores. The results indicate that 16 out of 18 patients (88.8%) improve ($n = 10$) or go into remission ($n = 6$) with the median PDAI score before and after therapy being 11 and 4 respectively ($P < 0.002$)[49].

More recently, 44 patients with refractory pouchitis received metronidazole (800 mg to 1 g/d) and ciprofloxacin (1 g/d) for 28 d. The results reveal that 66 patients (82%) go into remission with the median PDAI score before and after therapy being 12 and 3 respectively ($P < 0.0001$), and the patients' quality of life is significantly improved after the treatment (median IBD Questionnaire score increased from 96.5 to 175)[50].

Table 3 Organisms associated with probiotic activity

Most commonly	Other bacterial strains	Yeast
Lactobacilli	Enterococci	Saccharomyces boulardii
Bifidobacteria	Non-pathogenic *E. coli*	
Streptococci		

PROBIOTICS

The use of probiotics for the purpose of health maintenance and disease prevention is first proposed by Elie Metchnikoff, the Russian Nobel prize winner[51], who at the turn of the last century suggested that a high concentration of lactobacilli in the intestinal flora is important for the health and longevity of humans. Probiotics are defined as "living organisms, which upon ingestion in a certain number exert health benefits beyond inherent basic nutrition"[52].

A number of bacteria are associated with probiotic activity (Table 3). For clinical application, probiotic strains need to be resistant to acid and bile and the ability to be metabolically active within the luminal flora where they should ideally survive but not persist in the long term. They should be antagonistic to pathogenic bacteria and safe for human use while maintain their viability and beneficial properties during the manufacturing processes[53].

Animal model studies

Encouraging results of probiotic therapy have been obtained in experimental colitis. Administration of *Lactobacillus reuteri* can significantly reduce inflammation in acetic acid-and methotrexate-induced colitis in rats[54,55]. More recently *Lactobacillus sp.* has been shown to be able to prevent the development of spontaneous colitis in IL-10 deficient mice[56], and continuous feeding with *Lactobacillus plantarum* improves an established colitis in the same knockout model[57]. A strain of *Lactobacillus salivarius* (subsp. *salivarius*) reduces the rate of progression from inflammation to dysplasia and colonic cancer in IL-10 deficient mice[58], and *Bifidobacterium infantis* and of *Lactobacillus salivarius* are able to attenuate inflammation and reduce the ability to produce Th1-type cytokines in the IL-10 knockout model[59].

VSL#3 is characterised by a very high bacterial concentration (each packet containing 450 billion viable bacteria) and the presence of a cocktail of eight different bacterial species. This product contains viable lyophilised bacteria of four strains of lactobacilli (*L. casei, L. plantarum, L. acidophilus, L. delbrueckii* subsp. *bulgaricus*), three strains of

bifidobacteria (*B. longum, B. breve, B. infantis*) and one strain of *Streptococcus salivarius* subsp. *Thermophilus*. Rachmilewitz and colleagues[60] found that VSL#3 results in a significant attenuation of inflammation with a decrease of myeloperoxidase and nitric oxide synthase activity of the iodoacetamide-induced colitis. Madsen and colleagues[61] have reported a significant improvement in inflammation together with a reduction in mucosal levels of pro-inflammatory cytokines and normalisation of colonic barrier integrity in IL-10 knockout mice.

Ulcerative colitis

Promising results of probiotics have been found in the treatment of UC. In 3 recent trials involving the non-pathogenic strain of *Escherichia coli* Nissle 1917, similar efficacy has been observed to that of mesalazine in the maintenance treatment of UC[62-64].

We carried out a pilot study using the probiotic cocktail, VSL#3, as maintenance treatment for patients with UC in remission, allergic or intolerant to sulphasalazine and mesalazine, to assess its impact on the faecal flora. Twenty patients received 6 g a day of VSL#3 (1800 billion bacteria) for 12 mo and were assessed clinically and endoscopically at baseline, at 6 and 12 mo, and in the event of a relapse. Stool culture and determination of faecal pH were also performed at different intervals[65]. Microbiological determination showed a significant increase in concentration of lactobacilli, bifidobacteria and *Streptococcus thermophilus,* evident after just 20 d, which persisted throughout the treatment period, and returned to basal levels within 15 d after treatment. Faecal concentration of *Bacteroides*, enterococci, coliforms, *Clostridia* and total anaerobes and aerobes was not affected, but faecal pH was significantly reduced by the treatment. Fifteen of the twenty patients (75%) remained in remission throughout the treatment period[65].

Furthermore, VSL#3 at very high dosage (3600 billion bacteria/d) can induce remission in 63%, with a positive response in a further 23% of patients with active mild to moderate disease[66].

In addition, an open uncontrolled 4-wk study found that the yeast *Saccharomyces boulardii* could induce remission in 71% of patients with mild to moderate UC[67]. These studies highlight the wide range of organisms that may be beneficial as probiotic therapy for UC.

Crohn's disease

Campieri *et al*[68] performed a randomised trial to evaluate the efficacy of a combination of rifaximin and the probiotic preparation, VSL#3, in the prevention of post-operative recurrence of CD. Rifaximin (1.8 g/d) for 3 mo, followed by VSL#3 (6 g daily) for 9 mo, was compared with mesalazine (4 g/d) for 12 mo in 40 patients after curative resection for CD. After 3 mo of treatment, the antibiotic-probiotic combination resulted in a significantly lower incidence of severe endoscopic recurrence compared to mesalazine [2/20 (10%) *vs* 8/20 (40%)]. This difference was maintained throughout the study period [4/20 (20%) *vs* 8/20 (40%)][68].

No such clinical effect was seen in a study by Prantera *et al*[69] who reported that the probiotic *Lactobacillus GG* could not prevent post-operative disease recurrence in an 1-year double-blind, placebo-controlled trial. Similar negative results have been recently reported by the GETAID French group. A randomised double-blind, placebo-controlled study showed that *Lactobacillus johnsonii* LA1 ($4x10^9$ cfu/d) is not superior to placebo in preventing endoscopic recurrence of CD[70].

In a small pilot study[71], treatment with capsules containing *E.coli* Nissle 1917 was compared to placebo in the maintenance of steroid-induced remission of colonic CD. Twelve patients were treated with *E.coli* Nissle and 11 with placebo. The results showed that at the end of the 12-wk treatment period, the relapse rate is 33% in the *E.coli* group and 63% in the placebo group. Unfortunately, because of the small number of patients treated, this difference did not reach statistical significance.

However, a small comparative open study[72] showed that the combination of *Saccharomyces boulardii* (1 g/d) and mesalazine (2 g/d) is significantly superior to mesalazine (3 g/d) in maintenance of remission, suggesting that probiotic treatment in CD may be beneficial. More recently, a double-blind trial showed that *Lactobacillus* GG is not superior to placebo in prolonging remission in children with CD when given as an adjunct to standard therapy[73].

Pouchitis

Although probiotics are less widely used in clinical practice than antibiotics, they may be efficacious in the prevention and treatment of pouchitis. We have compared the efficacy of VSL#3 with placebo in the maintenance and treatment of chronic pouchitis[74]. Forty patients who obtained clinical and endoscopic remission after 1 mo of combined antibiotic treatment (rifaximin 2 g/d + ciprofloxacin 1 g/d) were randomised to receive VSL#3, 6 g daily (1800 billion bacteria/d) or a placebo of identical appearance for 9 mo. Clinical assessment was carried out every month, endoscopic and histological assessments were performed at entry and subsequently every two months. Stool samples were cultured before and after antibiotic treatment and subsequently every month during maintenance treatment. Relapse was defined as an increase of at least 2 points in the clinical portion of the PDAI and confirmed endoscopically and histologically. Whilst all 20 patients treated with placebo had a relapse during the 9 mo follow-up period, 17 of the 20 (85%) patients treated with VSL#3 remained in remission at this point. Interestingly, all these 17 patients had a relapse within 4 mo after the active treatment. Faecal concentrations of lactobacilli, bifidobacteria and *Streptococcus salivarius* subsp. *thermophilus* were significantly increased within 1 mo after VSL#3 treatment, and remained stable throughout the study. However, this increase did not affect the concentration of the other bacterial groups, suggesting that the beneficial effect of treatment is not mediated by suppression of endogenous luminal bacteria.

A recent study examining the maintenance of remission in patients with refractory or recurrent pouchitis showed that remission is achieved in 85% of patients treated with VSL#3, 6 g/d (1800 billion bacteria/d) and 6% in

the placebo group after 1 year of treatment[75]. In addition, continuous administration of VSL#3 results in a significant increase in IL-10 tissue levels, a significant decrease in tissue levels of the pro-inflammatory cytokines (TNF alpha, IL-1 and IFN gamma) and a decrease in matrix metalloproteinase activity[76]. This may aid our understanding of the mechanisms of action by which VSL#3 maintains remission in pouchitis. In contrast, *Lactobacillus GG* is ineffective in preventing relapse in patients with chronic pouchitis[77].

We have also carried out a double-blind, placebo-controlled trial to evaluate the efficacy of VSL#3 in preventing pouchitis onset following ileal-anal anastomosis for UC[76]. Forty patients were randomised to receive VSL#3, 3 g per day (900 billion bacteria/d) or an identical placebo for 12 mo. Patients were assessed clinically, endoscopically and histologically at 1, 3, 6, 9 and 12 mo according to the PDAI. The results indicate that patients treated with VSL#3 have a significantly lower incidence of acute pouchitis compared with those treated with placebo during the first year of ileostomy closure (10% *vs* 40%; $P < 0.05$). Moreover, the IBD Questionnaire score is significantly improved in the group treated with VSL#3, and the median stool frequency in patients not developing pouchitis, is significantly less in the VSL#3 group compared with the placebo group[78].

CONCLUSION

There is strong evidence that enteric commensal bacteria are involved in the pathogenesis of IBD. Therefore, modification of the gut bacterial flora by antibiotics and probiotics may be effective in treating UC, CD and pouchitis.

Antibiotics are a well established, efficacious treatment option for various manifestations of IBD. Antibiotics play an essential role in treating the septic complications of CD, including intra-abdominal and perianal abscesses and perianal fistulae, although their use in CD as a primary therapy is poorly documented. There is good evidence that ciprofloxacin, metronidazole or their combination is effective in Crohn's colitis and ileocolitis, though not in isolated ileal disease. Large controlled trials are needed to define optimal antibiotic regimens. In addition, their use in UC is not supported by the available studies and large trials with broad-spectrum agents are required. Although proper controlled trials have not yet been conducted, the use of antibiotics in pouchitis is largely justified.

Probiotics provide an attractive alternative to antibiotics in the treatment of IBD as trials to date have shown that they safe and have no side-effects. Promising results have been obtained from studies using probiotics, in both the prevention of relapse and the treatment of mild to moderate attacks of UC. Studies using probiotics in the treatment of CD are less clear due to conflicting and limited data. There is also considerable evidence that the highly concentrated cocktail of probiotics, VSL#3 is efficacious in preventing pouchitis onset and relapse.

Studies have highlighted the importance of selecting a well characterised probiotic preparation for treatment. In fact, viability and survival of bacteria in many available preparations are unproven. It should be remembered that the beneficial effect of one probiotic preparation does not imply efficacy of other preparations containing different bacterial strains, because each individual probiotic strain has its unique biological properties.

There is a need to improve our understanding of the composition of the enteric flora and the relationship between intestinal physiology and the luminal ecosystem. Only then can we truly optimise the bacteria-modifying treatments now available to effectively treat IBD.

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REVIEW

Consensus of primary care in acute pancreatitis in Japan

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Abstract

The incidence of acute pancreatitis in Japan is increasing and ranges from 187 to 347 cases per million populations. Case fatality was 0.2% for mild to moderate, and 9.0% for severe acute pancreatitis in Japan in 2003. Experts in pancreatitis in Japan made this document focusing on the practical aspects in the early management of patients with acute pancreatitis. The correct diagnosis of acute pancreatitis and severity stratification should be made in all patients using the criteria for the diagnosis of acute pancreatitis and the multifactor scoring system proposed by the Research Committee of Intractable Diseases of the Pancreas as early as possible. All patients diagnosed with acute pancreatitis should be managed in the hospital. Monitoring of blood pressure, pulse and respiratory rate, body temperature, hourly urinary volume, and blood oxygen saturation level is essential in the management of such patients. Early vigorous intravenous hydration is of foremost importance to stabilize circulatory dynamics. Adequate pain relief with opiates is also important. In severe acute pancreatitis, prophylactic intravenous administration of antibiotics at an early stage is recommended. Administration of protease inhibitors should be initiated as soon as the diagnosis of acute pancreatitis is confirmed. A combination of enteral feeding with parenteral nutrition from early stage is recommended if there are no clear signs and symptoms of ileus and gastrointestinal bleeding. Patients with severe acute pancreatitis should be transferred to ICU as early as possible to perform special measures such as continuous regional arterial infusion of protease inhibitors and antibiotics, and continuous hemodiafiltration. The Japanese Government covers medical care expense for severe acute pancreatitis as one of the projects of Research on Measures for Intractable Diseases.

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Key words: Fluid resuscitation; Protease inhibitor treatment; Antibiotic treatment; Continuous regional arterial infusion; Contrast-enhanced computed tomography

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INTRODUCTION

There are several guidelines for the treatment of acute pancreatitis, mostly based on published evidence[1-4]. If, however, there were no published evidence, data would not be adopted in guidelines even if the therapy is employed in clinical practice. There is little evidence for the treatment of acute pancreatitis in critically early stage, and thus it is not mentioned in the guidelines.

In July 2003, the Japanese Society for Abdominal Emergency Medicine, the Japan Pancreas Society and the Research Committee of Intractable Diseases of the Pancreas supported by the Japanese Ministry of Health, Labour, and Welfare published "Evidence-Based Clinical Practice Guidelines for Acute Pancreatitis"[2,5]. The working party of the Research Committee of the Intractable Diseases of the Pancreas made the present document for the management of acute pancreatitis in the early stages after onset[6], which was not mentioned in the guidelines[2,5]. The management of patients with acute pancreatitis is complicated by an unpredictable outcome[7]. Many patients initially identified as having mild disease progress to severe disease indolently over the initial 48 h. This document is the consensus of experts in pancreatitis in Japan and focuses on the practical aspects in the early management of patients with acute pancreatitis and decisions needed within the first 48 h of care, introducing literatures on the treatment of acute pancreatitis published in Japanese.

This consensus guideline on acute pancreatitis is a review of literatures and experts' opinions in Japan intended for general clinicians in primary care and emergency department, and gastroenterologists who do not have expertise in pancreatitis. However, this consensus by experts is only an example of standard practical medical care for patients with acute pancreatitis in Japan. Physicians need not necessarily follow this consensus but have to determine measures depending on hospital and patient specifics.

ESSENTIALS OF PRIMARY CARE OF ACUTE PANCREATITIS

All patients diagnosed with acute pancreatitis should be managed in the hospital. Monitoring of blood pressure, pulse and respiratory rate, body temperature, hourly urinary volume, and blood oxygen saturation level (SpO_2) by pulse oximetry is essential. Early vigorous intravenous hydration is of foremost importance. The correct diagnosis of acute pancreatitis and severity stratification should be made in all patients using the criteria for the diagnosis of acute pancreatitis and the multifactor scoring system proposed by the Research Committee of Intractable Diseases of the Pancreas[8] as early as possible. Following the correct diagnosis of acute pancreatitis, severity stratification should be performed promptly and repeatedly after the onset, in particular for the first 48 h.

It is not possible to distinguish mild from severe acute pancreatitis during the early stages. Serum levels of amylase and lipase do not reflect the severity of acute pancreatitis[9,10]. Even patients who appeared to be mild acute pancreatitis at the first hospital visit (within 48 h after onset) often deteriorate. All patients with severe acute pancreatitis must be monitored closely for the development of organ failure and should be managed in, or referred to, an intensive care unit (ICU) with full monitoring and systems support. The mortality rate of patients with mild acute pancreatitis within the first 24 h who later showed exacerbation during 24-48 h was reported to be 11%[11].

When the diagnosis of severe acute pancreatitis was made, systemic administration of antibiotics and protease inhibitors, and special measures such as continuous regional arterial infusion (CRAI) of protease inhibitors and antibiotics, continuous hemodiafiltration (CHDF), and selective decontamination of the digestive tract (SDD) should be considered.

Patients with severe acute pancreatitis and those requiring interventional radiological, endoscopic, or surgical procedures should be managed in, or referred to, a specialty unit. Principles of management of acute pancreatitis are summarized in Table 1 and Figure 1.

DIAGNOSIS OF ACUTE PANCREATITIS

Acute pancreatitis should be diagnosed according to the diagnostic criteria for acute pancreatitis proposed by the Research Committee of Intractable Diseases of the Pancreas[8]. Clinical criteria for the diagnosis of acute pancreatitis are (1) acute abdominal pain and tenderness in the upper abdomen, (2) elevated pancreatic enzyme levels in blood, urine, or ascitic fluid, and (3) radiologic abnormalities characteristic of acute pancreatitis. Acute pancreatitis can be diagnosed when two or more of the above criteria are fulfilled and other causes of acute abdominal pain are excluded. Acute exacerbation of chronic pancreatitis is included in acute pancreatitis.

The incidence of acute pancreatitis in Japan appears to be increasing[12] and ranges from 187 to 347 cases per million populations. Approximately 4.9% of patients who visited hospitals complaining of acute abdominal pain were found to have acute pancreatitis[13]. Thus, acute pancreatitis should be considered as a differential diagnosis for cases with signs and symptoms of digestive diseases. A history of the present illness and life-style, physical examination, blood tests such as amylase and lipase, and imaging studies such as abdominal plain X-ray, ultrasonography (US) and computed tomography (CT) should be undertaken to assist in the diagnosis of acute pancreatitis. Therefore, every hospital that receives acute admissions should have facilities for acute pancreatitis to be diagnosed at any time. In addition, physicians should be aware of the presence of acute pancreatitis patients but without abdominal pain or

Table 1 Principles of treatment for patients with acute pancreatitis at initial and early stage

Management	Physical check lists	Examination	Treatment
Within 24 h after onset			
Initial vigorous intravenous hydration Severity stratification Assessment of etiology All patients with severe acute pancreatitis should be transferred to a high special unit or intensive therapy unit.	Consciouness status Abdominal findings Blood pressure Pulse rate Respiratory rate Body temperature Urinary volume Pulse oximetry (SpO_2)	CBC and blood chemistry Chest and abdominal x-ray Abdominal US Abdominal CT and/or MRI Arterial blood gas analysis	Secure and maintain venous route Initial fluid resuscitation (60-160 mL/kg body weight/day) For the first 6 h, fluid resuscitation of about 1/2-1/3 of the amount required for the first 24 h Analgesics and oxygen, as required Protease inhibitors Antibiotics for severe cases and infection of the bile duct Consider CRAI CHDF in severe cases Urgent therapeutic ERCP or EST in patients with cholangitis or with disturbed bile flow (refer to a specialist unit where facilities and expertise are available for ERCP and EST)
From 24 to 48 h after onset			
Re-evaluation of severity All patients with severe acute pancreatitis should be transferred to a high special unit or intensive therapy unit.	Consciouness status Abdominal findings Blood pressure Pulse rate Respiratory rate Body temperature Urinary volume Pulse oximetry (SpO_2)	CBC and blood chemistry Chest and abdominal x-ray Arterial blood gas analysis Abdominal CE-CT, as required	Similar to the above-mentioned treatment In addition Correction of fluid resuscitation Enteral feeding in patients without clear signs and symptoms of ileus and gastrointestinal bleeding
After 48 h of onset			
Fundamental conservative therapy in moderate and mildcases All patients with severe acute pancreatitis should be transferred to a high special unit or intensive therapy unit.	Consciouness status Abdominal findings Blood pressure Pulse rate Respiratory rate Body temperature Urinary volume Pulse oximetry (SpO_2)	CBC and blood chemistry Chest and abdominal x-ray Arterial blood gas analysis Abdominal CE-CT, as required	Similar to the above-mentioned treatment In addition Correction of fluid resuscitation Enteral feeding in patients without clear signs and symptoms of ileus and gastrointestinal bleeding

SpO_2: Blood oxygen saturation level, CBC: Complete blood count; US: Ultrasonography; CT: Computed tomography, MRI: Magnetic resonance imaging; CE-CT: Contrast-enhanced computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy; CRAI: Continuous regional arterial infusion of protease inhibitors and antibiotics; CHDF: Continuous hemodiafiltration.

signs and symptoms.

The initial treatment should be started as early as possible following the diagnosis of acute pancreatitis.

PRIMARY CARE OF ACUTE PANCREATITIS (WITHIN 48 H OF DIAGNOSIS)

Severity stratification

Following the correct diagnosis of acute pancreatitis, severity stratification should be performed promptly and repeatedly after the onset, in particular for the first 48 h. Severity of acute pancreatitis should be evaluated and scored by the multifactor scoring system proposed by the Research Committee of Intractable Diseases of the Pancreas (JPN severity scoring system) (Table 2)[8]. We recognize that the Ranson, modified Glasgow, and APACHE II systems for evaluating acute pancreatitis are well established, but they might be outdated and are not used in Japan. Pathology-specific systems such as Ranson's and the modified Glasgow require 48 h of data collection before the severity can be evaluated. JPN severity scoring system contains variables that represent different organ systems and can be consistently recorded at the time of admission to facilitate immediate management decisions.

When the correct diagnosis of acute pancreatitis is made, it should be considered to perform contrast-enhanced CT (CE-CT) and/or CE-magnetic resonance imaging (CE-MRI)[14-17], systemic administration of antibiotics[18] and protease inhibitors[19], and special measures such as CRAI of protease inhibitors and antibiotics[20,21], CHDF[22], and SDD[23]. Patients with severe acute pancreatitis and those requiring interventional radiological, endoscopic, or surgical procedures should be managed in, or referred to, a specialty unit.

There is no simple and reliable index to predict aggravation of acute pancreatitis in the early stages. Despite the importance of recognizing severe disease early in the course, many patients initially identified as having mild disease progress to severe disease indolently over the initial 48 h[11]. Severity stratification should be made repeatedly, particularly within the first 48 h after admission. Early recognition of severe disease and application of

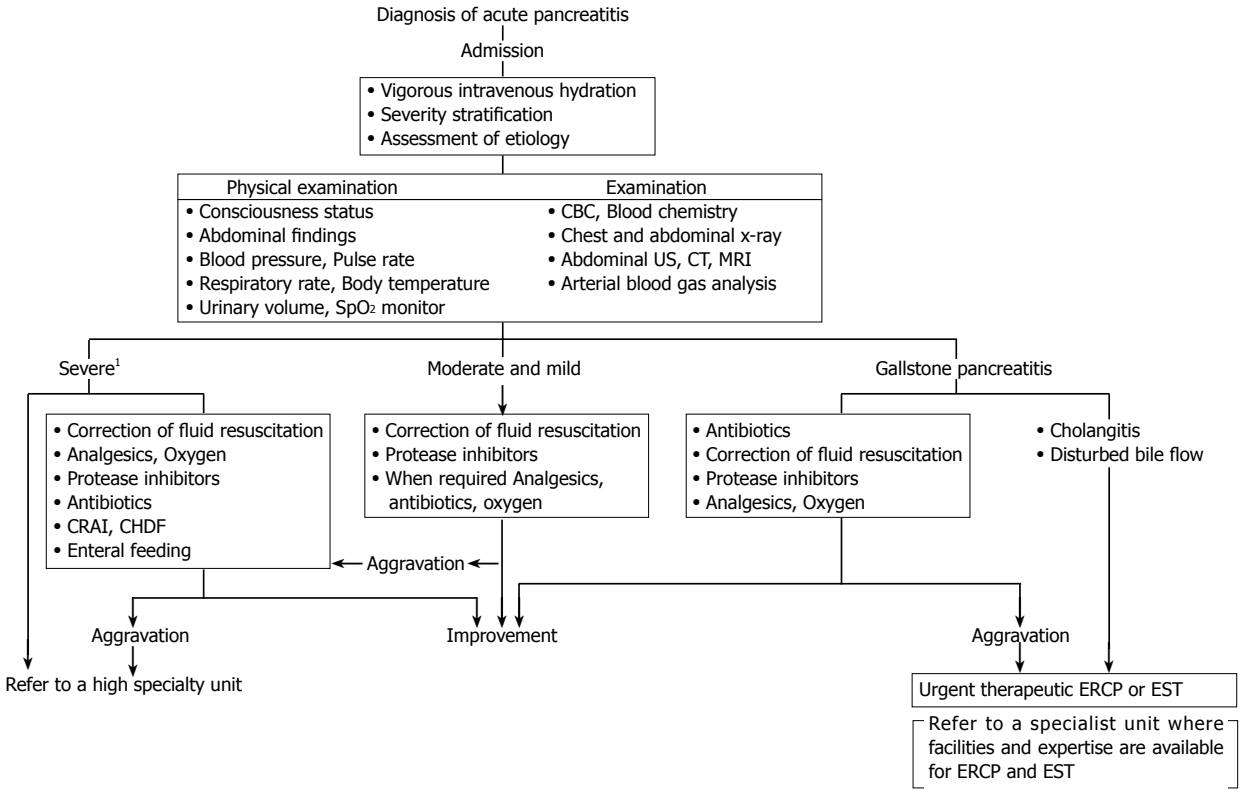


Figure 1 Algorithm of the primary management of acute pancreatitis at an early stage. [1]Hospitals that cannot provide appropriate monitoring and adequate systemic support should transfer all patients with severe acute pancreatitis to a high special unit or intensive therapy unit. SpO_2: Blood oxygen saturation level by pulse oximetry, CBC: Complete blood count, US: Ultrasonography, CT: Computed tomography, MRI: Magnetic resonance imaging, ERCP: Endoscopic retrograde cholangiopancreatography, EST: Endoscopic sphincterotomy, CRAI: Continuous regional arterial contrast infusion of protease inhibitors and antibiotics, CHDF: Continuous hemodiafiltration.

Table 2 Criteria for grading the severity of acute pancreatitis

Prognostic factors	Clinical signs	Laboratory data
Prognostic factor I (2 points for each positive factor)	Shock Respiratory failure Mental disturbance Severe infection Hemorrhagic diathesis	BE ⩽ -3 mEq/L Ht ⩽ 30% (after hydration) BUN ⩾ 40 mg/dL or creatinine ⩾ 2.0 mg/dL
Prognostic factor II (1 point for each positive factor)		PaO_2 ⩽ 60 mmHg (room air) FBS ⩾ 200 mg/dL Total protein ⩽ 60 g/L LDH ⩾ 700 IU/L Ca ⩽ 7.5 mg/dL Prothrombin time ⩾ 15 s Platelet count ⩽ 1×10^5 /mm^3 CT grade IV or V[1]
Prognostic factor III	SIRS score ⩾ 3 (2 points) Age⩾ 70 yr (1 point)	

BE: Base excess, Ht: Hematocrit, BUN: Blood urea nitrogen; FBS: Fasting blood sugar; LDH: Lactate dehydrogenase; SIRS: Systemic inflammatory response syndrome. [1]CT grade IV or V: Presence of diffuse and uneven density in the pancreatic parenchyma or the presence of inflammatory changes extending beyond the border of the pancreas. Severity score: Sum of the points for the positive prognostic factors is defined as the severity score. Standardized criteria: Severe: Presence of more than one prognostic factor I, and/or the presence of more than two prognostic factor II (severity score ⩾ 2 points). Moderate: Presence of one prognostic factor II (severity score = 1 point). Mild: Acute pancreatitis without prognostic factor I or II (severity score = 0 point).

Table 3 Stage classification of acute pancreatitis and mortality rate in nationwide survey in 2003 in Japan

Stage	Severity score	Severity	No of patients (%)[1]	Died[2]	Mortality rate
Stage 0	0 point	Mild	943 (53.3)	1	0.10%
Stage 1	1 point	Moderate	280 (15.8)	2	0.70%
Stage 2	2-8 points	Severe I	455 (25.7)	17	3.70%
Stage 3	9-14 points	Severe II	63 (3.6)	16	25.40%
Stage 4	⩾ 15 points	Most severe	27 (1.5)	16	59.30%
Total			1768 (100)	52	2.90%

[1]In 2004, nationwide survey of patients with acute pancreatitis who visited the hospitals in the year 2003 (from January 1 to December 31) was performed by stratified random sampling method. From the first survey, the total number of patients treated for acute pancreatitis in Japan in the year 2003 was estimated as 35 300 (95% confidence interval, 30 500-40 000). Clinical records of 1768 patients with acute pancreatitis were obtained in the second survey for analysis of etiology and outcome.
[2]Number of patients who died of acute pancreatitis or related complications.

appropriate therapy require vigilance as decisions regarding management need to be made shortly after admission, often within the first 24 h.

Case fatality in acute pancreatitis in Japan in 2003 according to the JPN severity score was 0.2% (3/1223) with 0-1 point (Stage 0, 1), 3.7% (17/455) with 2-8 points (Stage 2), and 35.6% (32/90) more than 9 points (Stage 3, 4) (Table 3)[12].

Assessment of etiology (diagnosis of acute gallstone pancreatitis)

The etiology of acute pancreatitis should be entertained

early, especially in patients with gallstone pancreatitis. Although the vast majority of stones that cause acute pancreatitis rapidly pass out of the common bile duct (CBD)[24], emergency endoscopic retrograde cholangiopancreatography (ERCP) with or without endoscopic sphincterotomy (EST) in selected patients with severe gallstone pancreatitis, who have evidence of biliary obstruction, cholangitis, and an elevated serum bilirubin levels within 24 h of admission, is reported to decrease the incidence of biliary sepsis[25]. Although abdominal US is considered the gold standard for the diagnosis of cholelithiasis, it is not sensitive for the evaluation of choledocholithiasis in the setting of acute pancreatitis. Abdominal US can detect gallstones in the gallbladder in 60%-80% of cases, and in the bile duct in 25%-90% of cases of gallstone pancreatitis in the early stage[26]. Gallstones may be present in the CBD even in the absence of biliary ductal dilatation on abdominal US[27]. Laboratory testing may assist in the early identification of CBD stones. In patients with serum alanine aminotransferase (ALT) (or glutamic-pyruvic transaminase [GPT]) values greater than 150 IU/L at admission and without a history of high quantities of alcohol intake, gallstone pancreatitis cannot be denied even in the absence of bile duct dilatation[27,28]. Increasing serum levels of bilirubin or transaminases within 24-48 h of admission for acute pancreatitis predicts a persistent CBD stone[27]. It is reported that combination of abdominal US and laboratory parameters enables to diagnose gallstone pancreatitis with a sensitivity of 94.9% and a specificity of 100%[24]. When the diagnosis of gallstone pancreatitis is in doubt, repeated imaging studies such as abdominal US, CT, MR cholangiopancreatography (MRCP)[29], or endoscopic ultrasound (EUS)[30], together with serum biochemical tests such as ALT, alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP) and total bilirubin may provide good evidence for the presence or absence of gallstone pancreatitis[28].

Urgent therapeutic ERCP or EST should be performed in acute pancreatitis patients with suspected or proven gallstones[24,31,32]. All patients with severe acute gallstone pancreatitis should be managed in, or referred to, a specialist unit where facilities and expertise exist for ERCP at any time for CBD evaluation followed by EST and stone extraction or stenting, as required.

Abdominal CT

Although it is not clear how soon the full extent of the necrotic process occurs and only a quarter of acute pancreatitis patients develop necrosis, it is current practice in Japan to perform CE-CT on admission for severity stratification and correct evaluation of the range of poorly perfused area. A low-density area (poorly perfused area) on CE-CT suggests ischemic change with vasospasm or necrosis in and around the pancreas[33]. The presence of pancreatic necrosis and its range, infiltration of inflammatory changes are closely related with severity and prognosis[15,34]. In addition, CE-CT is required for the decisions on special measures such as CRAI of protease inhibitors and antibiotics, and CHDF. However, since anxieties persist over the potential for extension of necrosis and exacerbation of renal impairment following the use of intravenous contrast media[35,36], vigorous intravenous hydration for the purpose of intravascular resuscitation is important during and after CE-CT.

In severe cases, CT evidence of a low-density area suggesting ischemic change with vasospasm or necrosis in and around the pancreas, free intraperitoneal fluid and extensive peripancreatic fluid collection are observed since an early stage. Since the full extent of the necrotic process occurs at least 4 d after the onset of symptoms[16] and an early CT may therefore underestimate the final severity of the disease, it is desirable to perform CT on admission and repeated CT for reevaluation 2 or 3 d later.

Evaluation and monitoring of circulatory dynamics and fluid resuscitation

Monitoring index: As the inflammatory process progresses early in the course of the disease, there is an extravasation of protein-rich intravascular fluid into the peritoneal cavity resulting in hemoconcentration. The reduced perfusion pressure into the pancreas leads to microcirculatory changes resulting in pancreatic necrosis[37]. At first, for appropriate fluid resuscitation, initial evaluation and monitoring of circulatory dynamics is important.

In all cases, status of consciousness, blood pressure, pulse and respiratory rate, oxygen saturation, body temperature, and hourly urinary volume should be monitored appropriately and chronologically.

The most important aim of fluid resuscitation is to stabilize circulatory dynamics, largely to keep the blood pressure and pulse rate at levels identical to those before onset of acute pancreatitis, and secure reasonable urinary volume. Since the urinary volume is closely related to appropriate circulatory plasma volume and blood pressure, maintenance of urinary volume is easy to monitor; 1 mL/kg body weight is the minimal hourly urinary volume that should be maintained.

In addition to the above-mentioned monitoring, cardiothoracic ratio (CTR) by chest X-ray, blood gas analysis, serum electrolyte, and hematocrit should be determined to perform appropriate fluid resuscitation. Despite sufficient initial fluid resuscitation, aggravation of consciousness status, and the occurrence and progression of metabolic acidosis require re-examinations of other factors such as mesenteric ischemia and reevaluation of circulatory dynamics by determining central venous pressure (CVP) and monitoring pulmonary-artery catheter pressure. In cases of unstable circulatory dynamics, transfer to ICU with full monitoring and systems support should be considered.

Fluid resuscitation: (1) The amount of fluid resuscitation. Extravasation of protein-rich intravascular fluid into the peritoneal cavity early in the course of acute pancreatitis causes hemoconcentration and intravascular hypovolemia leading to impaired microcirculation in the pancreas. The decreased blood flow leads to stasis, thrombosis and subsequent pancreatic necrosis. Vigorous intravenous hydration can prevent the development of pancreatic necrosis. Fluid resuscitation for acute pancreatitis should be started by extracellular fluid such as acetate or lactate Ringer solution from a peripheral vein. Healthy adults require 1500-2000 mL fluid (30-40 mL/kg body weight),

whereas patients with acute pancreatitis require 2-4 times (60-160 mL/kg body weight) the volume administered to healthy adults.

The severity of acute pancreatitis changes at every moment even if it appears to be mild on admission. Although acute pancreatitis typically results in significant intravascular losses, patients with acute pancreatitis are often given suboptimal fluid resuscitation. Since it is rare for the administration of large fluid volumes to be problematic in mild cases, aggravation by insufficient fluid resuscitation must be avoided. In particular, a large amount of fluid resuscitation (about 1/2-1/3 of the amount required for the first 24 h) is necessary in the first 6 h. Approximately 6 h after initiation of treatment, blood pressure, pulse rate, hourly urinary volume and the above-mentioned index must be re-evaluated to make plan for subsequent fluid resuscitation. In a mild case of acute pancreatitis, the amount and rate of fluid resuscitation should be reduced.

If shock or a pre-shock is present on arrival at the hospital, rapid intravenous fluid resuscitation of 500-1000 mL must be performed. Patients of 60 kg body weight with moderate to severe acute pancreatitis require about 1200-4800 mL fluid resuscitation for the first 6 h and 3600-9600 mL for the first 24 h. If the patient does not recover from circulatory failure by fluid resuscitation, administration of catecholamine and transfer to ICU with full monitoring and systems support should be considered.

We have reported that the average amount of fluid resuscitation on the first hospital day in patients who died of acute pancreatitis was 2788 ± 246 mL (n = 52, mean ± standard deviation) and that the amount of fluid resuscitation in 78.9% of these fatal cases for the first 24 h after admission was less than 3500 mL[38]. Indeed, all patients with inadequate fluid resuscitation (rehydration less than 4.0 liters) as evidenced by persistence of hemoconcentration at 24 h developed necrotizing pancreatitis[39].

The amount of fluid resuscitation required on the first and the following hospital day in moderate acute pancreatitis to maintain 120 mmHg or higher systolic blood pressure and hourly urinary volume of more than 1 mL/kg body weight was 4873 ± 2280 mL/d and 2000-2500 mL/d, respectively, while that in severe acute pancreatitis was 7787 ± 4211 mL/d and 4000-5000 mL/d, respectively[40]. Based on abdominal CT findings, Takeda *et al*[41] reported that the amount of fluid resuscitation on the first hospital day in patients with acute pancreatitis with inflammatory changes extending to anterior pararenal space, mesentery of the colon and the retroperitoneum beyond inferior pole of the kidney was approximately 4000 mL, 6000 mL, and 8000 mL, respectively. In some cases, body weight may increase more than 10 kg within a few days after onset of acute pancreatitis. It will take 1-2 wk for diuresis to occur resulting from re-filling to decrease body weight[41].

A large amount of fluid resuscitation is necessary until an adequate urinary volume is attained. Administration of diuretics before achievement of adequate fluid resuscitation may lead to a deterioration of the situation. Restriction of the amount of fluid resuscitation and excessive diuresis by diuretics because of fear of aggravation of pulmonary and systemic edema by a large amount of fluid resuscitation at this time may exacerbate the dysfunction of important organs including the kidney. Fluid resuscitation should be performed to promote prompt recovery from a potential shock state even if respiratory care under intratracheal intubation is required as a result of a large amount of fluid resuscitation. (2) Route of infusion. It is desirable to secure and keep the central venous route to maintain long-term fluid resuscitation and continuous infusion of protease inhibitors in moderate and severe acute pancreatitis, while the central venous route is redundant in mild cases. In severe acute pancreatitis, a central venous route should also be secured to monitor CVP chronologically and to determine the amount and rate of fluid resuscitation.

In the early stages of acute pancreatitis, vascular permeability often increases, and thus circulatory volume decreases resulting in a tendency for collapsed vascular lumen. Since access of the central vein is difficult in such cases, adequate levels of fluid should be given from a peripheral vein as the initial therapy. However, the femoral vein should be avoided if possible to prevent deep venous thrombosis and infection, and to securely obtain the route for CHDF and CRAI therapy. (3) Caution of fluid resuscitation. Since excessive amounts and a high rate of fluid resuscitation may cause pulmonary edema in patients with acute pancreatitis complicating heart, lung and renal dysfunction, and in elderly people, it is important to control the amount and rate of infusion according to the above-mentioned indexes such as blood pressure, pulse rate, urinary volume and CVP. However, it is thought that shock and aggravation of acute pancreatitis at an early stage tend to be due to insufficient amounts of the initial fluid resuscitation. It should be kept in mind to give adequate amount of fluid resuscitation at an early stage.

Serum levels of electrolyte and calcium should be measured and corrected appropriately, especially when tetany occurs due to low serum calcium concentrations.

There is no evidence to support the usefulness of administration of albumin preparation and fresh frozen plasma (FFP) for primary care within 48 h of onset. Administration of colloid fluid at the acute stage may retard improvement of pulmonary edema in the re-filling period.

Analgesics: Since acute pancreatitis often causes extreme abdominal pain, which may disturb cardiopulmonary functions, adequate pain relief is required.

The following drugs should be given; buprenorphine (0.1-0.2 mg intramuscular or intravenous injection, or 0.3 mg/h intravenous drip infusion) or pentazocine (7.5-15 mg intramuscular or intravenous injection, or 15-30 mg intravenous drip infusion). Since there is a possibility that these drugs induce spasms of the sphincter of Oddi and disturb the outflow of bile and pancreatic juice when given frequently, administration of atropine sulphate in combination with analgesics is recommended. In addition, these analgesics must be given with caution, due to the risk of drug dependence when used continuously. However, neither buprenorphine nor pentazocine is approved by health insurance in Japan for pain of acute pancreatitis. Intramuscular or subcutaneous injection of pentazocine and buprenorphine is only approved for pain control

of carcinoma and postoperative state, while intravenous injection of pentazocine is approved for anesthesia and buprenorphine for myocardial infarction and anesthesia.

A mixture of opium alkaloids and atropine (1.0 mL contains 20 mg opium alkaloid hydrochloride and 0.3 mg atropine sulphate, 1.0 mL subcutaneous injection), and a mixture of morphine and atropine (1.0 mL of this solution contains 10 mg morphine hydrochloride and 0.3 mg atropine sulphate, 1.0 mL subcutaneous injection) can also be given. Pain relief by epidural anesthesia is also possible in a high specialty unit. Since both reagents may cause respiratory depression, intensive monitoring of cardiopulmonary function is required after administration.

For mild pain in acute pancreatitis, non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac suppository and indomethacin suppository can be used. However, these agents should be used with caution due to associated adverse effects such as pernicious anemia, hemolytic anemia, granulocytopenia, thrombocytopenia, liver damage, acute renal failure, asthma, interstitial pneumonia, and peptic ulcer hemorrhage. These drugs are usually not used in moderate and severe cases of acute pancreatitis. In particular, NSAIDs are contraindicated in patients in pre-shock status.

Antibiotics: In mild and moderate cases of acute pancreatitis, prophylactic administration of antibiotics is unnecessary because the incidence of fatal infection of the pancreas and the exudates around the pancreas is low.

In severe acute pancreatitis, prophylactic intravenous administration of antibiotics at an early stage is recommended[18,42,43]. Antibiotics with a broad antibacterial spectrum and good penetration into the pancreas[44] such as imipenem[18,42] and meropenem[43] are desirable. If infection of the biliary tract is suspected, administration of the second or later generation cephalosporins is also recommended. Clinicians should always take microbial substitution and fungal infection into consideration when using antibiotics.

The maximum dose of antibiotics that is approved by the Japanese Government should be used to attain sufficient concentrations in and around the pancreas. For example, intravenous infusion of 1-3 g/d imipenem or meropenem dividing into 3 times is recommended (1.0 g of imipenem or meropenem can be dissolved in 100 mL physiologic saline or 5% glucose solution, and infuse for more than 30 min). However, special attention has to be paid to the patient's liver and renal function. In particular, in severe cases, it is necessary to re-evaluate the dose of antibiotics every day because liver and renal function changes chronologically day by day. If possible, it is recommended to monitor serum concentrations of antibiotics. Moreover, clinicians should be concerned about the possibility of developing fungal infections.

In cases with advanced inflammation in and around the pancreas, SDD is often undertaken to prevent bacterial translocation[23].

Protease inhibitors: Although the effect of protease inhibitors has some controversy, experts of pancreatitis in Japan recommend to administer protease inhibitors as soon as the diagnosis of acute pancreatitis is confirmed[19,45]. Recent meta-analysis of 10 articles of randomized controlled trials evaluating the effects of protease inhibitors for acute pancreatitis has revealed that treatment with protease inhibitors does not significantly reduce mortality in patients with mild acute pancreatitis, but may reduce mortality in patients with moderate to severe acute pancreatitis[46]. Table 4 shows examples of protease inhibitors used for acute pancreatitis in Japan[47].

Table 4 Recommended doses of protease inhibitors for acute pancreatitis[47]

	Mild and moderate	Severe
Protease inhibitor	FOY FUT UTI	FOY + UTI FUT + UTI
Initial dose[2] (during the first 12 h)	Maximum usual one-day dose[1] by continuous intravenous infusion	Maximum usual one-day dose[1] by continuous intravenous infusion
Until d 3[2]	Above dose for 24 h	Above dose for 24 h
First wk[2]	Gradually reduce the above dose or intermittent administration	Above dose for 24 h
Second wk[2]	Reduce the dose or cease	Maintain or gradually reduce the above dose
Third wk[2]	Reduce the dose or cease	Maintain or gradually reduce the above dose

FOY: Gabexate mesilate, FUT: Nafamostat mesilate, UTI: Ulinastatin. [1]Administer the one-day dose during the initial 12 h. [2]Severity stratification should be made repeatedly, and the doses of protease inhibitors must be increased or decreased depending on the severity. The usual daily dosage for acute pancreatitis of FOY, FUT and UTI approved by the health insurance is 200-600 mg, 10-60 mg and 50 000-150 000 units, respectively. Since, however, severe acute pancreatitis often complicates disseminated intravascular coagulation (DIC) and shock, it is recommended to give these reagents in doses that are approved for these disorders in severe acute pancreatitis (daily dosage for severe acute pancreatitis of FOY, FUT and UTI is 20-39 mg/kg body weight/d, 1.44-4.8 mg/kg body weight per day, and 10 000-300 000 units /d, respectively).

Usual doses of gabexate mesilate (FOY), nafamostat mesilate (FUT) and ulinastatin (UTI) for acute pancreatitis are 200-600 mg/d, 10-60 mg/d and 50 000-150 000 units/d, respectively. However, since severe acute pancreatitis is often complicated by disseminated intravascular coagulation (DIC) and shock, it is recommended that these reagents should be given in doses approved for these disorders in severe acute pancreatitis. The guidelines for acute pancreatitis in 1991 from the Research Committee of Intractable Diseases of the Pancreas recommended combination therapy with FOY or FUT together with UTI when severe pancreatitis is predicted[47].

FOY and FUT have several adverse effects that clinicians must be aware of; FUT increases serum potassium (K) concentration[48,49], and FUT and FOY may cause phlebitis, and inflammation, ulcer and necrosis of the skin with extravasation. In addition, there are reports of anaphylactic shock by repeated administration of FOY and FUT[50,51].

Enteral feeding: Based on the fact that enteral feeding stimulates pancreatic secretions[52], classical management recommends that patients presenting with acute pancreatitis be fasted until symptoms begin to resolve. However, there is no sufficient evidence to support the concept of gut rest (prohibiting enteral intake) in acute pancreatitis. Acute pancreatitis results in hypermetabolic and hyperdynamic situation creating a high catabolic stress state[53]. Failure to achieve a positive nitrogen

balance is associated with increased mortality in patients with severe acute pancreatitis[54]. Traditionally, total parenteral nutrition (TPN) has been advocated in these patients in order to supply adequate nutrients and prevent hypercatabolic states avoiding stimulation of exocrine pancreatic secretions. However, TPN in patients with acute pancreatitis has not been shown to be beneficial[55]. Early enteral nutrition through a nasojejunal tube is associated with a significantly lower incidence of infections, reduced surgical interventions, and a reduced hospitalization period compared with TPN[56]. Enteral nutrition is as safe and efficacious as TPN and may be beneficial during the clinical course of this disease. Even in severe acute pancreatitis, a combination of enteral feeding with parenteral nutrition from early stage is recommended if there are no clear signs and symptoms of ileus and gastrointestinal bleeding[58].

Although the majority of studies have reported enteral feeding via a nasojejunal tube, a recent pilot study of early nasogastric feeding in patients with severe acute pancreatitis proved that this approach might be feasible in up to 80% of cases[59]. However, caution should be used when administering nasogastric feeding to patients with impaired consciousness due to the risk of aspiration of refluxed feed.

Other treatments: Patients with acute pancreatitis often have deficiency of vitamin B1 due to alcohol abuse and malnutrition or abnormal and irregular dietary habits. Exacerbation of lactic acidosis or shock in such patients may suggest a deficiency of vitamin B1 and require corrective measures.

TRANSFER TO ANOTHER HOSPITAL

Transfer time to a high specialty unit

All patients when diagnosed with acute pancreatitis should be managed in a hospital. Patients with suspected acute pancreatitis should be examined at hospitals with facilities for blood examination and imaging studies. Depending on the severity, full monitoring and systemic support in high care unit (HCU) or ICU is necessary. Hospitals that cannot provide appropriate monitoring and adequate systemic support, should transfer all patients with severe acute pancreatitis to ICU as early as possible.

Evidence-based therapeutic guidelines for severe acute pancreatitis[2,5] published by the Japanese Society for Abdominal Emergency Medicine, the Japanese Pancreas Society and the Research Committee of Intractable Diseases of the Pancreas recommends transferring acute pancreatitis patients with the JPN severity score 2 points or above[8]. Severe or aggravating cases should be transferred to ICU as early as possible to perform the CRAI of protease inhibitors and antibiotics or CHDF.

Instructions of transfer

During transfer, cardiopulmonary function such as blood pressure, pulse and respiratory rate, and oxygen saturation must be monitored. In addition, a venous route must be secured by continuing fluid infusion to be able to cope promptly with a sudden change. In severe cases, a doctor and a nurse should accompany the patient during transfer.

Before deciding to transfer a patient, it should be considered about the influence of street transportation for long time on the patient's clinical condition when vigorous fluid resuscitation is necessary at early period of illness.

Local core hospitals require facilities that can deal with severe acute pancreatitis.

SPECIAL MEASURES

Continuous regional arterial infusion of protease inhibitors and antibiotics (CRAI)

To obtain high concentrations of protease inhibitors and antibiotics in the pancreas, these reagents are continuously infused into the artery through a catheter with tip placed at a portion where the bloodstream irrigates the inflamed area of the pancreas. Concentrations of protease inhibitors and antibiotics in pancreatic tissue after CRAI are approximately 5[60] and 5-10 times[61], respectively, higher than those after intravenous infusion. This procedure is therefore expected to directly inhibit inflammatory processes in the pancreas by strongly inhibiting activity of proteases that might aggravate pancreatic damage and inhibit most of the pathogens present in pancreatic infections[8,20,21,62]. Since protease inhibitors used in Japan also have anticoagulant and anticomplement activities [63], it is possible that these reagents inhibit the development of pancreatic necrosis by preventing the formation of microthrombi in pancreatic vessels and evolution of ischemia into pancreatic necrosis.

This procedure is indicated in severe acute pancreatitis cases with a low density area of the pancreas on CE-CT on admission, which corresponds with ischemic change and vasospasm on angiography or necrosis in and around the pancreas[33]. Takeda *et al*[21] have demonstrated that CRAI of protease inhibitors and antibiotics is effective in reducing mortality and preventing the development of pancreatic infection when initiated within 48 h after the onset of severe acute pancreatitis. FUT or FOY is used for this procedure, because these agents are synthetic low-molecular-weight protease inhibitors that inhibit a number of serine-proteases, and C1r and C1s esterases[63]. In addition, FOY and FUT might easily penetrate into the pancreatic acinar cells due to their low molecular weight and inhibit the inflammatory process in the pancreas. With regard to antibiotics, imipenem is used because of its broad spectrum and good penetration into pancreatic tissue[44]. Since CRAI of protease inhibitors and antibiotics for the treatment of acute pancreatitis is still debatable and not covered by health insurance in Japan, informed consent must be obtained from the patient before performing this procedure.

Continuous hemodiafiltration (CHDF)

CHDF is a combination of continuous hemofiltration with hemodialysis developed as a continuous renal replacement therapy for patients with severe conditions. It is usually performed continuously for 24 h, and for 3-14 d. This procedure can be used to control fluid balance in patients with excessive fluid resuscitation but inadequate diuresis. However, in some hospitals this procedure is positively performed even if there is sufficient urinary volume, because CHDF can remove excess humoral mediators

including cytokines during the hypercytokinemic state of systemic inflammatory response syndrome such as severe acute pancreatitis[22,64,65].

When this procedure is started early after onset of acute pancreatitis, reduction of chemical mediators and improvement of respiratory function is obvious, while the incidence of multiple organ failure is low compared to cases in which the procedure is initiated at a late stage[64]. Health insurance in Japan has approved CHDF as one of insurance-applicable treatment modalities for severe acute pancreatitis.

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GASTRIC CANCER

Relationships between mucinous gastric carcinoma, MUC2 expression and survival

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Abstract

AIM: To investigate the expression of the four secreted gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) in a series of gastric carcinomas, classified according Laurén's, Mulligan's, WHO and Goseki's classifications, with special attention to all the different components (major and minor) present in tumors and to follow up clinical data.

METHODS: Expression of MUC2, MUC5AC, MUC5B and MUC6 was investigated using immunohistochemistry and *in situ* hybridization.

RESULTS: Expression of secreted gel-forming mucins in gastric carcinoma was particularly complex, each mucin being not restricted to any histopathological type even considering all components (major and minor) present in a given tumor. There was a worst survival in patients with a higher content of mucus (Goseki II or IV) and high positive MUC2 expression.

CONCLUSION: Complexity of mucin gene expression patterns in gastric cancer may reflect a precise state of differentiation at the cell level not recognized in used morphologic classification systems. High expression of MUC2 was nevertheless associated with mucinous subtype of the WHO classification and with group II of Goseki's classification identified by the major component of a particular tumor. The quantity and quality of mucus were related to survival.

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Key words: Gastric cancer; Secreted gel-forming mucin; MUC; Immunohistochemistry; *in situ* hybridization; Survival

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INTRODUCTION

Gastric cancers constitute a highly heterogeneous group of tumors with respect to epidemiology, genetics, histopathology and biological behavior. Numerous histopathological classifications have been proposed to identify the morphological variability in a reproductible manner and to correlate to prognosis. These different classifications include Laurén's, Mulligan's, World Health Organization (WHO), and Goseki's classifications, which all are based at least in part on the quantity of mucus present in an individual tumor[1-3]. The Goseki's classification combines two distinct criteria, e.g. the tubular differentiation and the intra-cellular mucin content[3]. It was shown to be the only classification with prognostic value additional to TNM staging[4]. All histopathological classifications are based on the identification of the major component of the tumor, neglecting the various minor components. However, in pathological practice, assessment of a histopathological type of gastric carcinoma is difficult due to the considerable variation frequently present within an individual tumor.

Epithelial mucins consist of at least thirteen different types that are usually subdivided into secreted gel-forming mucins and membrane-bound mucins[5-7]. The first class includes the four large mucins MUC2, MU-

C5AC, MUC5B and MUC6 whose genes are clustered on chromosome 11p15.5 and which are involved in the mucus gel formation. MUC5AC and MUC6 are the major mucin components in normal gastric mucosa. MUC5AC is highly expressed in mucous cells of the superficial and foveolar epithelium, whereas MUC6 is present in mucous neck cells and in mucous glands of cardia and antrum[8-11]. MUC5AC and MUC6 are also expressed in some gastric carcinomas[12-15]. Moreover, MUC2 and MUC5B which are absent or barely detectable in normal gastric tissues have been reported in gastric carcinoma[12-16]. MUC2 expression has been related more specifically to the mucinous carcinomas of WHO classification and to group II of Goseki' s classication (high tubular differentiation and high mucin content) whereas MUC5AC has been associated with the group IV of Goseki's classification (poor tubular differentiation and high mucin content)[14,15,17]. However, coexpression of multiple mucins is frequently observed in gastric carcinomas[9,13,15]. Such a complex pattern of mucin expression may reflect the variety frequently observed in gastric carcinomas.

In an effort to bring additional information in understanding the histopathological diversity of gastric carcinomas, we investigated the expression of the secreted gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) in a series of gastric carcinomas with special attention to all the different components (major and minor) present in tumors and to follow up clinical data.

MATERIALS AND METHODS

Tissue samples and histopathological study

A series of 31 gastric adenocarcinomas was obtained from institutional files. Cases included 7 women and 24 men with a mean age of 65 years-old, from 39 to 89 years. The surgical specimens were quickly immersed in fresh 4 g/L neutral formaldehyde solution (pH 7.4) in phosphate buffer and then were processed for paraffin embedding. Each gastric carcinoma was classified according to each of the following histopathological classifications; i.e. Laurén's classification, Mulligan's classification, WHO classification and Goseki's classification. These classifications are based on the identification and classification of the major component of the tumor on hematoxylin-eosin-saffron (HES) coloration. The Goseki's classification was realized on an adjacent tumor slide with Periodic Acid Schiff and Alcian Blue combined coloration. The Goseki's classification combines two distinct criteria, e.g. the tubular differentiation and the intracellular mucin content[3]. Four group are defined: group I, high tubular differentiation and poor mucin content; group II, high tubular differentiation and high mucin content; group III, poor tubular differentiation and poor mucin content; group IV, poor tubular differentiation and high mucin content[3]. The classification proposed by Mulligan recognizes three forms of gastric carcinoma: the intestinal cell type, the mucous cell type and the pylorocardiac gland cell type. In this classification the intestinal and mucous cell types are similar to the intestinal and diffuse types defined by Lauren. The pylorocardiac gland cell type carcinoma is a distinct group of gastric carcinoma characterized by large cells with clear cytoplasm [2]. The differentiation of each tumor was assessed on the basis of the degree of gland formation as well, moderate, poorly differentiated or undifferentiated. When the tumor presented with a major and one or more minor components, a sample where the different components were present was chosen to further perform the immunohistochemical and *in situ* hybridization studies on serials sections (4-μm thick).

Immunohistochemistry

Immunohistochemistry was performed using an automated immunostainer (ES; Ventana medical systems, Strasbourg, France). Primary antibodies were directed against MUC2 (polyclonal, LUM2-3, 1/1000)[18], MUC5AC (monoclonal, 1/2) (Novocastra, New Castle, UK), MUC5B (polyclonal, Lum5B-2, 1/1000)[19], MUC6 (monoclonal, 1/5) (Novocastra). The pretreatment was realized by a steamer for MUC2 (for 1 min 30) or by a microwave for MUC5AC, MUC5B and MUC6 (for 20 min), in citrate buffer (pH 6.0). For polyclonal antibodies, the sections were incubated for 32 min with normal goat serum to block the non-specific antibody-binding sites and endogenous peroxidase activity was suppressed by first incubating the specimen in 30 mL/L hydrogen peroxide. The immunohistochemistry method used a 3-step indirect process based on the biotin-avidin complex. Slides were counterstained with hematoxylin. Negative controls were run by omission of the primary antibody and positive controls with the appropriate tissue (normal colonic mucosa for MUC2, normal gastric mucosa for MUC5AC and MUC6, normal respiratory submucosal glands for MUC5B). The immunohistochemical results were estimated on the whole tumor and on the different components. The immunostaining was scored semiquantitatively as: 0, no immunostained cells; 1, less than 30% of immunostained cells; 2, more than 30% and less than 60% of immunostained cells; 3, strictly more than 60% of immunostained cells.

In situ hybridization

In situ hybridization was performed as described previously using four ^{35}S-labeled antisense oligonucleotide probes corresponding to each tandem repeat domain of *MUC2*, *MUC5AC*, *MUC5B*, and *MUC6*[20]. Briefly, tissue sections were deparaffinised, rehydrated, incubated with 2 mg/L proteinase K (Roche Diagnostics, Meylan, France) for 15 min and fixed again in 40 g/L paraformaldehyde in PBS for 15 min. Sections were then immersed in 0.1 mol/L triethanolamine (Sigma, L'Isle d'Abeau Chesnes, France) containing 2.5 ml/L acetic anhydride for 10 min. Sections were prehybridized in 4 × SSPE, 1×Denhardt' s buffer for 45 min, and hybridized overnight at 42 ℃ in 20-100 μL of a 4 × SSPE solution containing 500 mL/L formamide, 1 g/L *N*-lauroylsarcosine, 1.2 mol/L sodium phosphate (pH 7.2), 1 × Denhardt's buffer, 3 g/L yeast tRNA, 20 mmol/L dithiothreitol, and 125 MBq/L of ^{35}S-labeled oligonucleotide. After post-hybridization washes, slides were dipped in LM-1 emulsion (Amersham, Les Ulis, France), developed 1-3 wk after exposure, and counterstained with methyl green pyronin (Sigma). The following controls were performed: (1) competition studies by treatment of tissue sections with

Table 1 MUC expression in 31 gastric carcinomas stratified according to Laurén's classification, Mulligan's classification, WHO classification and Goseki's classification *n* (%)

Parameter	*n*	MUC2		MUC5AC		MUC5B		MUC6	
		+ IHC[1]	+ ISH	+ IHC	+ ISH	+ IHC	+ ISH	+ IHC	+ ISH
Laurén									
Intestinal	14	3 (21)	13 (93)	8 (57)	7 (50)	12 (86)	5 (36)	6 (43)	6 (43)
Diffuse	12	1 (8)	6 (50)a	9 (75)	8 (67)	9 (75)	3 (25)	5 (42)	3 (25)
Unclassified	5	3 (60)	4 (80)	3 (60)	1 (20)	3 (60)	1 (20)	1 (20)	2 (40)
Mulligan									
Intestinal cell	18	5 (28)	16 (89)	11 (61)	8 (44)	15 (83)	6 (33)	7 (39)	7 (39)
Mucous cell	13	2 (15)	7 (54)	9 (69)	8 (62)	9 (69)	3 (23)	5 (38)	4 (31)
Pylorocardiac	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
WHO									
Papillary	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Tubular	14	3 (21)	13 (93)	8 (57)	7 (50)	12 (86)	5 (36)	6 (43)	6 (43)
Mucinous	3	3 (100)[a]	3 (100)	2 (67)	0 (0)	2 (67)	0 (0)	0 (0)	1 (33)
Signet-ring cell	12	1 (8)	6 (50)[a]	9 (75)	8 (67)	9 (75)	3 (25)	5 (42)	3 (25)
Undifferentiated	2	0 (0)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)
Goseki									
Group I	11	2 (18)	10 (91)	5 (45)	4 (36)	9 (82)	2 (18)	5 (45)	4 (36)
Group II	5	4 (80)[b]	5 (100)	4 (80)	2 (40)	4 (80)	2 (40)	1 (20)	2 (40)
Group III	9	0 (0)	4 (44)	6 (67)	5 (56)	5 (56)	3 (33)	3 (33)	4 (44)
Group IV	6	1 (17)	4 (67)	5 (83)	5 (83)	6 (100)	2 (33)	3 (50)	1 (17)

[1] MUC2 positivity in more than 30% of tumoral cells. IHC: Immunohistochemistry; ISH: *In situ* hybridization. [a]$P<0.05$, [b]$P = 0.01$ *vs* other groups.

a large excess of unlabeled oligonucleotide identical to or distinct from the ^{35}S-labeled-probe; (2) verification of the absence of background by careful examination of non-epithelial structures (vessels, muscle, and connective tissue). The intensity of the hybridization signal was scored semiquantitatively as: 0, absent; 1, weak (visible at magnification ×200); 2, moderate (visible at magnification × 100); 3, strong (visible at magnification ×40).

Statistical analysis

Comparisons of the percentages in the different classifications were performed using chi-square and Fischer's exact tests. Survival was determined from the date of surgery to the time of death using the Kaplan–Meier method. Statistical significant of differences in survival curves was evaluated using the log-rank test. All statistical analyses were carried out by the SPSS 10.0 (SPSS, Chicago, IL, USA) software system. $P < 0.05$ were considered to indicate significant differences.

RESULTS

Histopathological results

Among the 31 gastric carcinomas, 2 were well differentiated, 11 moderately differentiated, 16 poorly differentiated and 2 undifferentiated. 14 carcinomas were classified as intestinal type in the Laurén's classification, these tumors being mainly classified as intestinal cell type in the Mulligan's classification, as tubular in WHO classification and as group I or II in the Goseki's classification. 12 tumors were classified as diffuse type in the Laurén's classification, being classified as mucous cell type in the Mulligan's classification, as signet-ring cell in the World Health Organization (WHO) classification and as group III or IV in the Goseki's classification. 3 carcinomas, not individualized in the Laurén's and Mulligan's classifications, corresponded to the mucinous type of the WHO classification and were classified in group II of Goseki's classification (Table 1).

Relationship between histopathological classifications and MUC expression

All MUCs were observed in each subtype of gastric carcinomas classified either in Laurén's classification, Mulligan's classification, WHO classification or Goseki's classification (Table 1, Figure 1). MUC2 was the major secreted gel-forming mucin detected in our series of gastric carcinomas. It was detected in 81% of the cases by immunohistochemistry and in 74% of the cases by *in situ* hybridization. MUC2 immunoreactivity was observed in the cytoplasm, in intracellular mucus vacuoles of tumoral cells, and also in extracellular mucus (Figure 1). Detection of MUC2 was not associated with a particular subtype of gastric carcinoma whatever the classification. However, high MUC2 immunostaining (in more than 30% of the tumoral cells), was significantly associated with the mucinous subtype of WHO classification ($P=0.015$) and with the group II of the Goseki's classification ($P=0.01$, Table 1). Although less significant, the absence of *in situ* hybridization signal for MUC2 was more frequent in the signet-ring subtype of the WHO classification ($P=0.04$) and in the diffuse subtype of Laurén's classification ($P=0.04$, Table 1). MUC5AC was detected in 65% of the cases by immunohistochemistry and in 52% of the cases by *in situ* hybridization. MUC5AC immunoreactivity was localized in the cytoplasm of tumoral cells, without staining of intracellular mucus vacuoles (Figure 1). MUC5AC detection either by immunohistochemistry or *in situ* hybridization was not associated with a particular subtype of gastric carcinoma classified by Laurén's, Mulligan's, WHO or Goseki's classifications (Table 1). MUC5B was

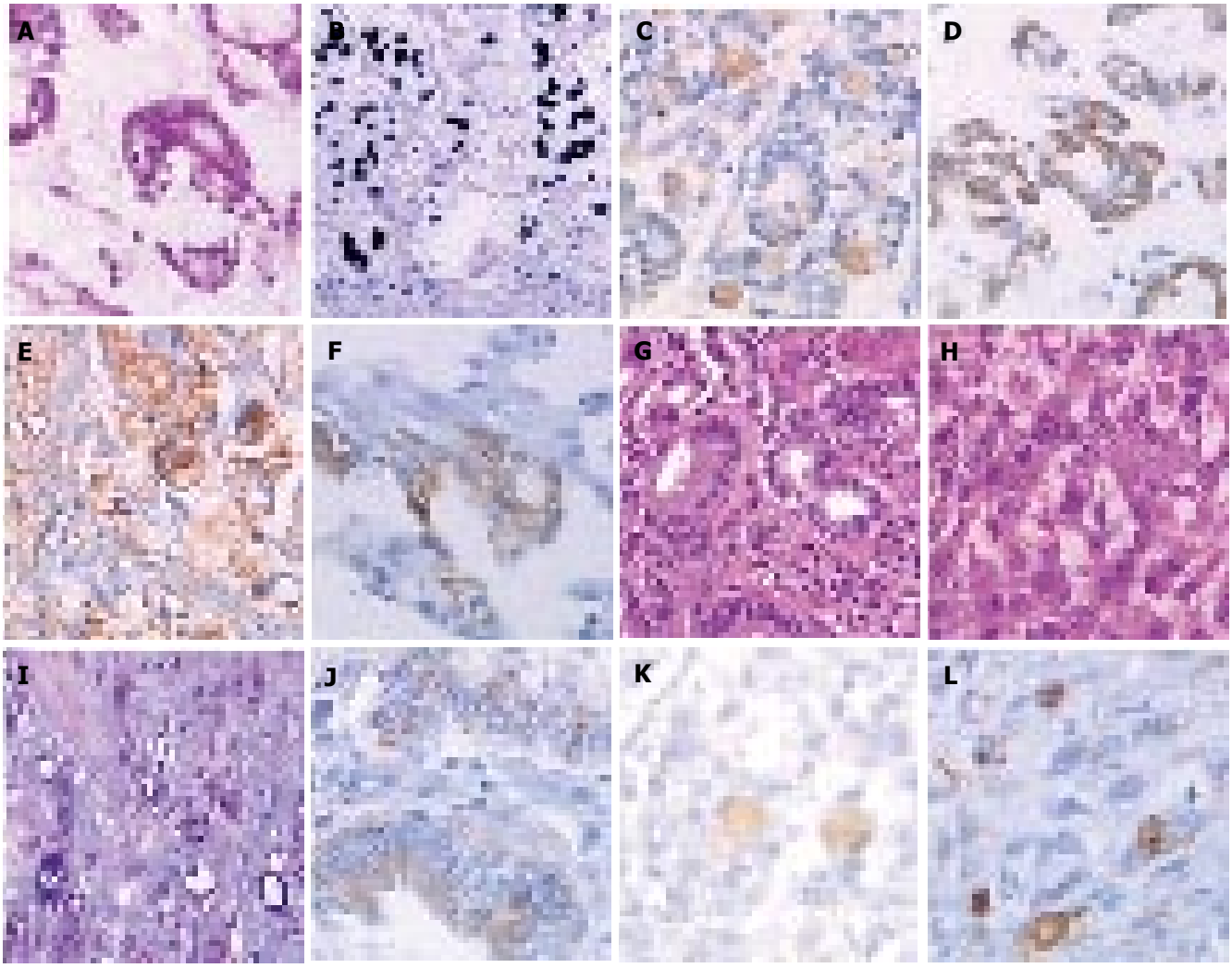


Figure 1 Expression of the secreted gel-forming mucins in gastric cancers. **A**: Mucinous subtype in WHO classification, group II in Goseki's classification (HE-safron × 125); **B:** Strong MUC2 in mucinous subtype (*in situ* hybridization × 40); **C**: MUC2 in intracellular mucus vacuoles in mucinous subtype (SABC × 125); **D:** MUC5AC in the cytoplasm of tumoral cells in mucinous subtype (SABC × 125); **E:** MUC5B in the cytoplasm, within intracellular mucus vacuoles and in extracellular mucus in mucinous subtype (SABC × 125); **F**: MUC6 in the cytoplasm of tumoral cells in mucinous subtype (SABC × 200); **G:** Gastric cancer classified group I in Goseki's classification (HE-safron × 125); **H:** Gastric cancer classified group III in Goseki's classification (HE-safron, × 200); **I:** Gastric cancer classified group IV in Goseki's classification (PAS and Alcian blue × 200); **J:** MUC2 in the cytoplasm of tumoral cells in group I gastric cancer Goseki's classification (SABC × 200); **K:** MUC2 in intracellular mucus vacuoles in group III gastric cancer in Goseki's classification (SABC × 200); **L:** MUC2 in intracellular mucus vacuoles in group IV gastric cancer in Goseki's classification (SABC × 200).

detected in 77% of the cases by immunohistochemistry and in 29% of the cases by *in situ* hybridization. MUC5B immunoreactivity was observed in the cytoplasm, in intracellular mucus vacuoles of tumoral cells and in extracellular mucus (Figure 1). MUC5B detection either by immunohistochemistry or *in situ* hybridization was not associated with a particular subtype of gastric carcinoma whatever the classification (Table 1). The discrepancies between the immunohistochemical and *in situ* hybridization results for MUC5B and at a lesser extent for MUC5AC corresponded mostly to cases which presented less than 30% of positive cells by immunohistochemistry and were negative by *in situ* hybridization. MUC6 was detected in 39% of the cases by immunohistochemistry and in 35% of the cases by *in situ* hybridization. The detection was observed in the cytoplasm of the tumoral cells, without staining of intracellular mucus vacuoles (Figure 1). Its expression was not associated with a particular subtype of gastric carcinoma whatever the classification (Table 1). No significant difference was observed for immunohistochemical or *in situ* hybridization detection of MUC2, MUC5AC, MUC5B or MUC6 between well/moderate differentiated carcinomas and poorly/undifferentiated carcinomas. The coexpression of at least two MUCs in the same tumor was frequent either identified by immunohistochemistry (83% of the gastric carcinomas) or *in situ* hybridization (54%). The coexpression of three MUCs in the same tumor was also remarkable either by immunohistochemistry (64%) or *in situ* hybridization (38%). In the series, 22% of the cases presented with the four MUCs detected by immunohistochemistry and 13% by *in situ* hybridization. The coexpression of at least two MUCs in the same tumor was not associated with a particular subtype of the

Table 2 MUC expression in each component of gastric carcinoma on the basis of Goseki's classification *n*(%)

Goseki,	*n*	MUC2		MUC5AC		MUC5B		MUC6	
		+ IHC	+ ISH	+ IHC	+ ISH	+ IHC	+ ISH	+ IHC	+ ISH
Group I	21	14 (67)	17 (81)	12 (57)	8 (38)	12 (57)	7 (33)	6 (29)	6 (29)
Group II	12	10 (83)	10 (83)	7 (58)	7 (58)	8 (67)	5 (42)	5 (42)	7 (58)
Group III	10	2 (20)	1 (10)	4 (40)	3 (30)	4 (40)	1 (10)	1 (10)	1 (10)
Group IV	13	9 (69)	6 (46)	7 (54)	5 (38)	7 (54)	2 (15)	4 (31)	2 (15)

IHC: Immunohistochemistry; ISH: *In situ* hybridization.

Goseki's classification.

Relationship between the different components and MUC expression

Among this series of 31 gastric carcinomas, we observed an histopathological heterogeneity in the majority of individual tumors. Indeed, 61% of the series presented with more than one component (one major component and one or more minor components). Given the difficulty to demonstrate a constant correlation between MUC expression and histopathological classifications, we further evaluated the expression of the four secreted gel-forming mucins independently in all the different components, major or minor, classified according to the Goseki's classification, frequently present in a same gastric carcinoma.

When the immunohistochemical labeling was studied separately in each component of each gastric carcinoma classified on the basis of the Goseki's classification, MUC2, MUC5AC, MUC5B and MUC6 were observed in all the different groups (group I to IV, Table 2). In particular, MUC2 was not restricted to the components classified as group II of Goseki (83% of histopathological components of group II were positive for the immunohistochemical detection of MUC2), but was also detected in components classified as group I (67%), group III (20%) or group IV (69%). Similarly, MUC5AC, MUC5B and MUC6 were detected in components classified as group I, group II, group III or group IV. The comparison between the tumors which presented with a component of Goseki group II (major or minor) and the tumors which presented without this component was not statistically different.

Relationship between TNM and MUC expression

Among this series of 31 gastric carcinomas, 5 were T1, 20 were T2, 4 were T3 and 2 were T4, 2 were Nx, 6 were N0, 15 were N1, 5 were N2 and 3 were N3. The immunohistochemical and *in situ* hybridization detection of MUC2, MUC5AC, MUC5B or MUC 6 was not associated with T1-T2 tumors when compared to T3-T4 tumors, and was not different between N0-N1 tumors in comparison to N2-N3 tumors. Moreover, the detection of two or three MUCs in the same tumor, either by immunohistochemistry or *in situ* hybridization, was not significantly associated with T1-T2 or T3-T4 tumors, nor with N0-N1 or N2-N3 tumors.

Relationships between TNM, Goseki classification, MUC2 expression and survival

Follow up data showed that there was a significant difference in mean survival time decreasing between the carcinoma patients with T1, patients with T2, patients with T3 and those with T4 tumors (Log rank 9.63, $P=0.022$). There was also a significant difference in mean survival time decreasing between the carcinoma patients without lymph node metastasis (N0) and those with lymph node metastasis (N+) (Log rank 9.29, $P=0.0023$). Patients with Goseki II or IV classes had worst survival (mean 24 mo) than those with Goseki I or III classes (mean 51 mo) $P=0.0036$, Figure 2. Patients with positive immunohistochemical MUC2 expression more than 30% had a worst survival (mean 20 mo) than those with positive MUC2 expression less than 30% (mean 39 mo) $P=0.0434$ and those without MUC2 expression 0% (mean 72 mo) $P=0.0391$. Patients with positive immunohistochemical expression of MUC2 less than 30% had worst but not statistically significant, survival than those without MUC2 expression ($P=0.0951$, Figure 3).

DISCUSSION

In the present study, we looked for a correlation between the expression of the secreted gel-forming mucins MUC2, MUC5AC, MUC5B, MUC6 and histopathological classifications (WHO, Laurén, Mulligan and Goseki) in a series of 31 gastric carcinomas with a special attention to all components (major or minor) present in an individual tumor. Expression of MUC2 was frequently observed in all subtypes of gastric carcinoma whatever the classification. However, the high expression of MUC2 was significantly associated with the mucinous subtype of the WHO classification and with the group II of Goseki's classification (high tubular differentiation and high mucin content). A relationship between gastric mucinous carcinomas and group II of their classification has been previously reported by Goseki *et al*[3]. The WHO and the Goseki's classifications are more precise than the Laurén's and the Mulligan's classifications to classify gastric carcinoma, individualizing the mucinous subtype and group II respectively. Moreover, the Goseki's classification, that combine two distinct criteria, e.g. the tubular differentiation and the intra-cellular mucin content was shown to be the only classification with prognostic value additional to TNM staging[3,4]. The asso-

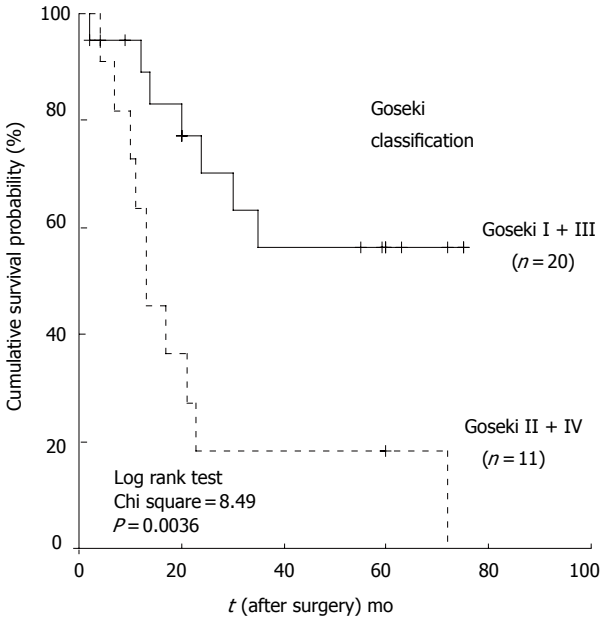


Figure 2 Survival curves for patients divided according to their Goseki classification.

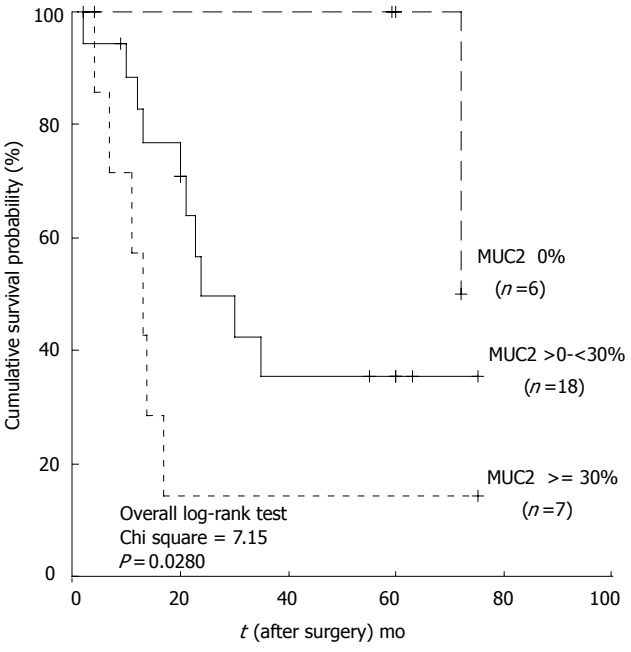


Figure 3 Survival curves for patients divided according to their immunohistochemical MUC2 expression.

ciation between expression of MUC2 and mucinous carcinoma has been previously reported in stomach, as well as in other organs, e.g. colon, breast, pancreas, ovary and pseudomyxoma peritonei[14-15,21-23]. Surprisingly, expression of MUC2 was not significantly associated with the intestinal subtypes of Laurén's or Mulligan's classifications ($P \geq 0.07$), although it is abundantly expressed in small intestine and colon as well as in intestinal metaplasia[8,24].

MUC2 is not the only secreted gel-forming mucin expressed in gastric carcinoma. Some gastric carcinomas expressed MUC5AC and/or MUC6, which are present in normal gastric mucosa[8,11]. Moreover, some gastric carcinomas expressed MUC5B, which was shown previously to be expressed during the stomach embryogenesis[25]. A significative association between the tumoral expression of MUC5AC and diffuse gastric carcinoma as defined in Laurén classification has been observed by some authors in comparison to unclassified carcinomas[14,26]. However, this association was controversed by others using the same MUC5AC antibody but comparing the diffuse subtype to the intestinal and unclassified carcinomas of the Laurén's classification[12,15]. MUC5AC has been also found more frequently expressed in group IV of Goseki's classification, which corresponds in part to the diffuse type of the Laurén's classification[3,15]. In our series, the expression of MUC5AC was frequent in the diffuse subtype of the Laurén's classification (75% of the cases in this subtype), in the group IV of Goseki' s classification (83%) and in the signet-ring subtype of the WHO classification (75%) but the comparison with the other subtypes did not reach the significant level. Moreover, the expression of MUC6 in our series was not associated with any histological type of the gastric carcinomas, whatever the classification, in accordance with previous studies [12-15]. In addition, expression of MUC5B, which before this study had never been extensively studied in gastric carcinoma using a morphological approach, was not found to be associated with particular subtypes of the Laurén's, Mulligan's, WHO or Goseki's classifications.

Given the difficulty to demonstrate a constant correlation between MUC expression and histopathological classifications, we further evaluated the expression of the four secreted gel-forming mucins independently in all the different components, major or minor, frequently present in a same gastric carcinoma. In our series, 39% of the tumors presented one component, whereas 61% presented at least two different components further classified according to the Goseki's classification. With this approach, none of the gel-forming mucin was restricted to any group of Goseki. In particular, MUC2 was not restricted to the components classified as group II but was also expressed in components classified as group I, III or IV. Furthermore, the comparison between the tumors which presented with a component of Goseki II (major or minor) and the tumors which presented without this component was not statistically different. Thus the association between MUC2 and the group II of Goseki was not reinforced by the study of each component. The best correlation between MUC expression and histopathological classifications was achieved for MUC2 and mucinous carcinomas of WHO classification and group II of Goseki's classification, these classifications being based on the identification of the major component of the carcinoma.

In this study, we also looked for a correlation between TNM and MUC expression. We did not found any association between expression of MUC (individual MUC or several MUCs) and T or N stade. More particularly, although increased mucin gene heterogeneity has been described in advanced gastric carcinomas[9], this was not confirmed in our series. A correlation between the

expression of gastric mucins, i.e. MUC5AC and MUC6 and a poorer prognosis has been reported by some authors[27]. Nevertheless, these results were not confirmed by others[17,28]. As expected, we observed a significant difference in mean survival time between the carcinoma patients according to the TNM stage. We observed a shorter mean survival time for patients with gastric carcinoma classified as Goseki II or IV in comparison to patients with Goseki I or III tumors. Interestingly, we also observed a shorter mean survival time for patients with positive MUC2 expression (in more than 30% of the cells) in comparison to patients without MUC2 immunohistochemical detection. Thus the quantity and quality of mucus present in gastric carcinoma were related to survival. This association between MUC2 expression and poorer outcome was controversed in the literature[17,29,30].

In conclusion, expression of secreted gel-forming mucins in gastric carcinoma is particularly complex. Complexity of mucin gene expression patterns in gastric cancer may reflect a precise state of differentiation at the cell level not recognized in the used morphologic classification systems. Nevertheless, this study showed a correlation between a high expression of MUC2 and mucinous subtype of gastric carcinoma that is achieved by considering the major component of the tumor. We also observed a shorter mean survival time in patients with a higher content of mucus (Goseki II or IV) and high positive MUC2 expression.

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LIVER CANCER

Reversing multidrug resistance by RNA interference through the suppression of MDR1 gene in human hepatoma cells

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Abstract

AIM: To reverse the multidrug resistance (MDR) by RNA interference (RNAi)-mediated MDR1 suppression in hepatoma cells.

METHODS: For reversing MDR by RNAi technology, two different short hairpin RNAs (shRNAs) were designed and constructed into pGenSil-1 plasmid, respectively. They were then transfected into a highly adriamycin-resistant HepG2 hepatoma cell line (HepG2/ADM). The RNAi effect on MDR was evaluated by real-time PCR, cell cytotoxicity assay and rhodamine 123 (Rh123) efflux assy.

RESULTS: The stably-transfected clones showed various degrees of reversal of MDR phenotype. Surprisingly, the MDR phenotype was completely reversed in two transfected clones.

CONCLUSION: MDR can be reversed by the shRNA-mediated MDRI suppression in HepG2/ADM cells, which provides a valuable clue to make multidrug-resistant hepatoma cells sensitive to anti-cancer drugs.

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Key words: Multidrug resistance; ShRNA; MDR1; Hepatocellular carcinoma

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, especially in Asia and Africa, with an estimated 0.5 million new cases and around 1 million deaths annually[1]. MDR is the most common impediment to successful chemotherapy for a variety of cancers[2], especially HCC. Classic MDR characterized by cross resistance to antineoplastic drugs, is caused by over-expression of MDR1 gene encoding P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) transporter superfamily. In liver, the expression of P-gp on the apical side of various cell membranes enhances the secretion of drugs into bile[3]. A disruption of P-gp-mediated drug extrusion results in a resensitization of tumor cells to treatment with antineoplastic agents, and may allow a successful drug treatment of the multidrug resistant cancer cells.

Most efforts at reversing MDR during the past two decades have been focused on compounds that modulate p-gp activity. In clinical trails, the efficiency of these drugs are difficult to access, mainly because of inherent adverse pharmacokinetic side effects[2], such as hypotension, heart failure, hyperbilirubinemia, and immunosuppression by cyclosporin A. Moreover, tumor cells can acquire resistance to the applied chemosensitizers, a so-called tertiary resistance. Consequently, it is necessary to develop alternative, less toxic and more efficient strategies to overcome MDR. It may, therefore, be more appropriate to target MDR1 gene expression. Indeed, MDR1 transcription has been targeted with ecteinascidin 743 in pre-clinical studies[4] and more recently by modulation of the orphan nuclear receptor SXR[5]. Strategies involving antisense and transcriptional decoy[6] and the use of anti-MDR1 mRNA hammerhead ribozymes have also been suggested [7].

RNAi is a conserved cellular mechanism by which double-stranded RNA (dsRNA) silences the corresponding homologous gene[8]. These 21-25 nucleotides with long, double-stranded small interfering RNA (siRNA) molecules can direct degradation of eukaryotic mRNAs in a sequence-specific manner. Two major discoveries have highlighted the potential of RNAi application in gene therapy. The introduction of synthetic siRNA of 21 nucleotides into mammalian cells can result in efficient gene silencing[9], and shRNAs expressed from RNA promoters within expression vectors also trigger RNAi[10, 11]. Recently, modulation of MDR by transfection of synthetic siRNAs has been reported [12, 13]. However, complete reversal to the drug-sensitive phenotype of parental cells has not been

obtained in any study. This is probably due to two reasons. Firstly, the transfection of synthetic siRNAs causes only transient suppression of target genes, which is often limited to the cell lines that are easy to be transfected. Secondly, the P-gp has a long half life time[14]. Thus, for achieving stable long-term RNAi effects, shRNA is developed[10].

In this study, we constructed two shRNAs targeting different coding regions of human MDR1 gene, because not all siRNA target sequences are equally potent[15]. After two shRNAs were respectively introduced into a multidrug resistant cell line HepG2/ADM derived from HepG2, efficiency of MDR1 gene suppression and whether MDR was reversed were evaluated.

Table 1 Sequences of shRNA oligonucloetides

Oligonucloetides	Sense + loop + antisense DNA template
MDR-A	5′-GATCCGCTTTGGCTGCCATCATCCAttcaagacgTGGATGATGG CAGCCAAAGTTTTTTGTCGACA-3′; 3′-GCGAAACCGACGGTAGTAGGTaagttctgcACCTACTACCGT CGGTTTCAAAAAACAGCTGTTCGA-5′
MDR-B	5′-GATCCGGCCTAATGCCGAACACATttcaagacgATGTGTTCGGCATTAGG CCTTTTTTGTCGACA-3′; 3′-GCCGGATTACGGCTTGTGTAaagttctgcTACACAAGCCGTAATCCGGAA AAAACAGCTGTTCGA-5′

The underlined parts are loops of shRNA oligonucleotides.

MATERIALS AND METHODS

Cell lines and cell culture

The HepG2 cells (Chinese Center for Type Culture Collection) were cultured in DMEM (Gibco BRL, Gaithersburg, MD, USA) contianing 10% of heat-inactivated FCS (Gibco BRL, Gaithersburg, Md. USA). Cells were incubated at 37 ℃ in a 50 mL/L CO_2 air incubator with saturated humidity. The establishment of a multidrug-resistant cell line HepG2/ADM was described previously[16]. The HepG2/ADM cells could survive a 24 h-treatment with adriamycin at a concentration of 5.0 mg/L, and are able to keep growing in the medium containing 1.0 mg/L adriamycin. To maintain MDR phenotype, HepG2/ADM cells were cultured with DMEM supplemented with adriamycin at 1.0 mg/L (Pharmacia, Uppsala, Sweden).

Design of siRNAs and cloning of siRNA hairpin loops

Two different siRNAs targeting different parts of the MDR1 gene (accession number: M14758) were designed: MDR-A (5'-AACTTTGGCTGCCATCATCCA-3', targeting nucleotide 586-606 of MDR1 mRNA sequence) was described previously [17]; MDR-B (5'- AAGGCCTA-ATGCCGAACACAT-3', targeting nucleotide 3494-3514 of MDR1 mRNA sequence) was designed according to the recommendation from the website http://sirna.qiagen.com. The two sequences were individually incorporated into a pair of oligonucleotides (produced by Wuhan GenSil Biotechnology, China. Table 1) where the target sequence appears as antisense followed by sense orientations separated by a 9-nucloetide spacer sequence and flanked at either end by Hind III or BamH1 restriction enzyme sites. The annealed oligonucleotides were cloned into pGenSil-l (Wuhan GenSil Biotechnology, China) according to the manufacturer's recommendations. The transcribed shRNA of MDR-A was 5'ATCCAttcaagacgTG-GATGATGGCAGCCAAAGU- -U-3', and the transcribed shRNA of MDR-B was 5'-GGCCTAATGCCGAACA-CATttcaagacgAT- -GTGTTCGGCATTAGGCCUU-3'. The underlined parts were loops of the two shRNAs.

Transfection and selection of stably transfected cell clones

The HepG2/ADM cells were transfected with 2μg of pGenSil-1/MDR-A, pGenSil-1/MDR-B, or empty vector pGenSil-1. Transfection was performed in 50%-60% confluent cells in 6-well plates using 9 μL of Lipofectamine 2000™ (Invitrogen, USA). Forty-eight hours after transfection, cells were grown in 10 cm cell plates with medium containing G418 (400 μg/mL) but without adriamycin. After 3 wk of culture, visible colonies were picked up and expanded. The stably transfected clone cells were observed to show green fluorescence under microscope and the clones without expression of the transfected gene did not show green fluorescence (Figure 1).

Detection of P-gp Function

Rh123 accumulation is sensitive and specific for indicating the transport function of P-gp[18, 19]. To observe the RNAi efficiency in the transfected cells, Rh123 (Sigma, MO, USA) was chosen to detect P-gp functions. The assay was performed as described by Broxterman *et al* [18]. Briefly, cells were cultured in 6-well plates. When the cells reached 70%-80% confluence, Rh123 was added to cells at a final concentration of 0.25 μg/mL and incubated at 37 ℃ for 1 h. The cells were washed 3 times with 4 ℃ PBS and resuspended at $5\text{-}10 \times 10^5$ cells/mL in 4 ℃ PBS. Rh123 fluorescence was analyzed with a FACStar flow cytometer (Becton Dickinson Immune Cytometry Systems) equipped with an argon laser. The blast population was gated by forward- and side-scatter characteristics. Rh123 fluorescence of 10 000 cells was measured logarithmically through a 530 nm bandpass filter at an excitation wavelength of 488 nm. HepG2 cells without incubation with Rh123 served as a negative control. Rh123 efflux was measured by counting cells in the M1 region of the plot and calculated as percentages of cells in the M1 region of the plot. The bigger percentage of cells in M1 region indicated the greater cellular Rh123 efflux and also a higher P-gp function. All 146 clones were checked.

Real-time reverse transcription chain reaction

Human MDR1 real-time PCR primers used in experiments are 5R-TGGTTCAGG TGGCTCTGGAT-3R and 5R-CTGTAGACAAACGATGAGCTATCA -CA-3R. Human GAPDH primers for real-time PCR used have been previously described[20]. Total RNA was isolated from the cells by TRIzol (Invitrogen, USA) 60-70 d after cell transfection. All RNA samples were stored at -80 ℃ until use. RNAs were reversely transcribed into cDNAs using RevertAid-TM first strand cDNA synthesis kit (Fermentas, Inc.) according to the manufacturer's instructions. Real-time PCR and data collection and result analysis were performed on

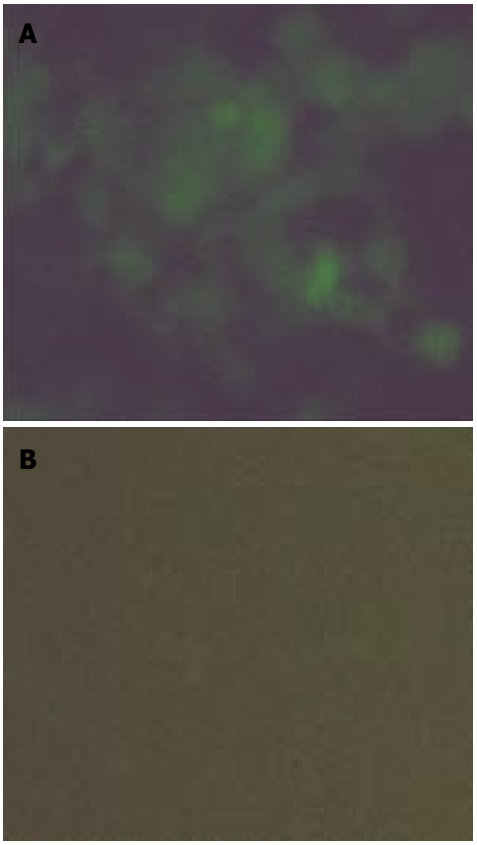


Figure 1 Stably transfected cells showing green fluorescence under a fluorescence microscope (**A**) and cells without stable expression of the transfected gene (**B**).

ABI PRISM®7000 sequence detection system (Applied Biosystem, USA). All quantitations were normalized to an endogenous control GAPDH. The relative quantitation value of each target gene was analyzed using a comparative C_T method. The following formula was used to calculate the relative amount of the transcript in the sample and normalized to an endogenous reference (GAPDH): $2^{-\triangle\triangle CT}$, where $\triangle C_T$ is the difference in C_T between the gene of interest and GAPDH, and $\triangle\triangle C_T$ for the sample = $\triangle C_T$ of the actual sample -$\triangle C_T$ of the lowest expressing sample (used as calibrator). The MDR1/GAPDH ratio in drug sensitive HepG2 cells was set at 1, and $L0g_{10}1 = 0.00$.

Cell cytotoxicity assay

The cytotoxicity assay and ID50 determination of anticancer drugs on the different clone cells were analyzed by the microculture methyl tetrazolium (MTT) method as described previously [21]. Briefly, in each experiment 10 000 cells per well were seeded in 96-well plates and cultured with DMEM or DMEM complemented with adriamycin in dilution series for 48h. Four repeated wells were used for each concentration. The total medium volume of each well was 200 μL. After 48 h of culture, 50 μL of (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, MO, USA) at 2 mg/mL was added to each well and incubated for 4 h at 37 ℃. Plates were centrifuged at 800r/min for 6 min, the medium was aspirated from plates, leaving about 30 μL of medium in each well. One hundred and fifty μL of dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany) was added to each well and the plates were shaken for 10 min to solubilize the formazan crystals. The plates were read immediately at 540 nm on a scanning multi-well spectrophotometer (Titertek, Alabama, USA). Absorbance from the cells treated with adriamycin was corrected against the absorbance from the untreated control cells. The ID50 value was defined as the dosage of drugs at which 50% of cells died after 48 h of treatment, which was calculated from 3 independent experiments for each clone. Since the basal growth rates of the cell lines had no significant difference in the first 2 d (data not shown), the errors caused by that were ignored.

Statistical analysis

For statistical analysis of P-gp function and cell cytotoxicity assay, the unpaired *t*-test was performed on SPSS 11.0 software. The real-time PCR data were analyzed using the SDS software on the ABI PRISM®7000 sequence detection system, the confidence limit was set at 95%.

RESULTS

ShRNAs decreased P-gp function

The RNAi efficiency was evaluated by measuring specific P-gp activity. P-gp-mediated transport indicated by intracellular decrease of Rh123 fluorescence was studied using flow cytometry. The P-gp activities of 58 pGenSil-1/MDR-A-transfected clones and 78 pGenSil-1/MDR-B-transfected clones were analyzed. Meanwhile, 10 HepG2/ADM control clones transfected with an empty pGenSil-1 vector were also analyzed. Two of the pGenSil-1/MDR-B-transfected clones showed a complete suppression of the P-gp function. More than 60% of the pGenSil-1/MDR-B-transfected clones (49 of 78 clones) showed an intermediate decrease of the P-gp function, whereas 8 clones did not show any significant changes of P-gp function. None of the pGenSil-1/MDR-A-transfected clones showed a complete suppression of P-gp activity, although different suppressions of P-gp function were detected. None of the control clones exhibited any significant alterations of P-gp function. Compared with HepG2 cells, the P-gp functions of HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells were at a low level near to that of the HepG2 cells, the P-gp functions of the HepG2/ADM cells and control HepG2/ADM/vec clone cells were much higher (Figure 2).

ShRNAs decreased MDR1 mRNA level

To further confirm the data from P-gp function study with Rh123 fluorescence, real-time RT-PCR was performed to quantify the RNAi-mediated suppression of MDR1 mRNA. The MDR1 mRNA expressions of parental cells, HepG2/ADM cells, HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells and the HepG2/ADM/vec control clone cells were measured. Expression of MDR-B shRNA in HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 reduced MDR1 mRNA expressions by 93% and 92%, respectively. MDR1 mRNA expression was reduced by 40% in the HepG2/ADM/vec control clone (Figure 3).

ShRNAs reversed multidurg-resistant phenotype

The reversal of MDR-mediated by shRNA was measured

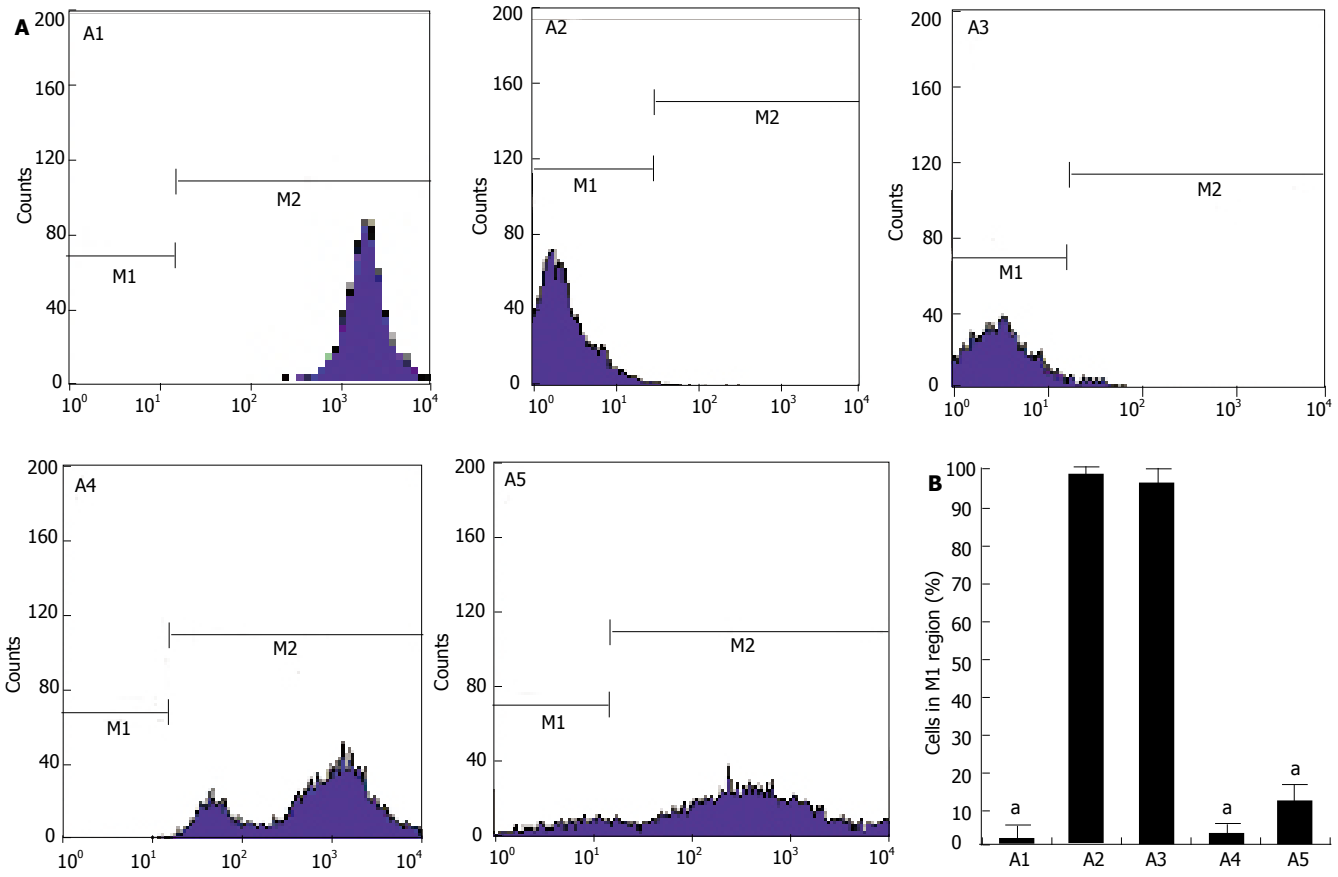


Figure 2 P-gp activity in HepG2 cells (A1), HepG2/ADM cells (A2), HepG2/ADM/vec control clone cells (A3); HepG2/ADM/pGenSil-l/MDR-B clone 1 cells (A4); HepG2/ADM/pGenSil-l /MDR-B clone 2 cells (A5) Rhodamine123 efflux was measured by counting cells in the M1 region of the plot. The marker bar M1 was set to indicate the cells with high Rhodamine123 efflux; the marker bar M2 was set to indicate the cells with low rhodamine123 efflux. Data were presented as mean ± SD. $^aP < 0.05$ *vs* untreated multidrug-resistant cell line HepG2/ADM.

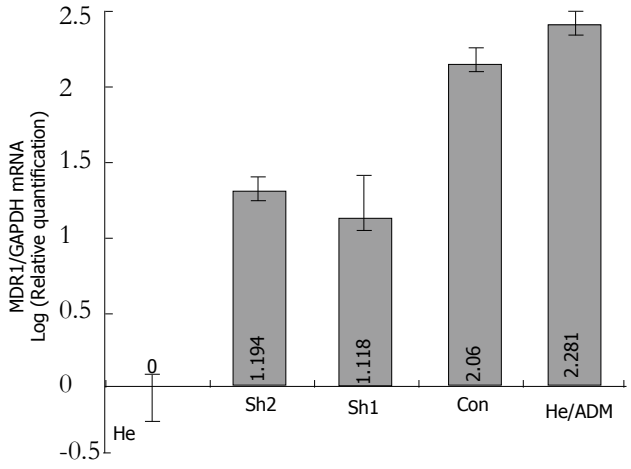


Figure 3 shRNA-mediated suppression of MDR1 mRNA. He, sh1, sh2, con and He/ADM represent HepG2, HepG2/ADM/pGenSil-l/MDR-B clone 1, HepG2/ADM/pGenSil-l/MDR-B clone 2, HepG2/ADM/vec control clone and HepG2/ADM, respectively. The relative expression values were the mean of triplicate real-time PCR reactions. Bars indicate SE.

using cell cytotoxicity to access adriamycin-specific ID50 levels. The resistance of HepG2/ADM to adriamycin was 102-fold stronger than that of the parental cell line at ID50 (Table 2). The HepG2/ADM/vec control clone had a similar resistance to HepG2/ADM cells. The resistance

Table 2 Effect of stable expression of shRNAs-mediated drug resistance to adriamycin

Cell line	Adriamycin IC50 (mg/L)	Resistance index (RI)
HepG2	0.141 ± 0.008	1
HepG2/ADM	14.394 ± 0.263[a]	102.085
Con	13.275 ± 0.353[a]	94.149
sh1	0.269 ± 0.035	1.908
sh 2	0.467 ± 0.617[a]	3.312

$^aP < 0.05$ *vs* ID50 value of HepG2. The data are presented as mean ± SD. Sh1, sh2 and con represent HepG2/ADM/pGenSil-l/MDR-B clone 1, clone 2, and the control HepG2/ADM/vec clone, respectively.

of HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells was decreased to a very low level near to that of the drug-sensitive HepG2 cells.

DISCUSSION

To improve the effect of RNAi-mediated gene silencing, some vector plasmids have been developed to express siRNAs as inverted repeats showing similar potency to initial RNAi. These siRNA-like molecules are commonly designated as shRNAs. For generating shRNAs, these vectors have been designed to be able to obtain the stable expression of shRNAs using strong RNA polymerase-II-depen-

dent promoters like CMV or EF1a, or RNA polymerse-III promoters like U6 or H1[10, 22-27]. Since experiences with antisense and ribozyme technology indicate that RNA polymerase-III expression systems can offer a great potency for maintaining stable expression of short RNA molecules *in vitro* as well as *in vivo*[28], the U6-driven expression vector pGenSil-1 was applied in this study. The size of the loop structure of shRNA has a considerable influence on the gene suppression activity. Brummelkamp *et al* [10] reported that a 9- nucleotide loop shows more effects than a 7-nucleotide loop, whereas a 5-nucleotide loop structure shows only moderate effects. Therefore, the two shRNAs were designed with a 9-nucleotide loop in our study.

The two shRNAs in this study showed different RNAi efficiencies. PGenSil-1/MDR-A was selected as previously described by Yague *et al* [17]. In his report, MDR-A shRNA constructed in pSUPER plasmid had an excellent RNAi effect on KD30 cell line, a multidrug resistant cell line derived from K562 leukemia cells. On the contrary, MDR-A shRNA did not show any significant RNAi effect on HepG2/ADM cells. One reason may be that the KD30 cell line is generated by a single-step selection in a low concentration of adriamycin (0.0174 mg/L)[17], while the HepG2/ADM cells are resistant to a high concentration of adriamycin. Another reason may be that the same siRNA sequence may have different RNAi efficiency in different tumor cells[12]. The different vector and loop structure of shRNA may also affect RNAi effects. Our study indicated that the pGenSil-1/MDR-B had a significant RNAi effect on HepG2/ADM cells. The MDR1 mRNAs were reduced by 93% and 92% respectively in HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 by expressions of MDR-B shRNA. The remaining MDR1 mRNA in the two clones was probably due to transgenic deletion, incomplete or inappropriate integration effects. The two clones showed a complete reversal of multidrug-resistant phenotype, indicating that the MDR-B siRNA might be very useful in designing new strategies for treatment of hepatoma in the future.

The empty pGenSil-l vector-transfected clone showed a slight decrease of MDR1 gene expression, which was suggested by real-time RT-PCR, Rh123 efflux test and cell cytotoxicity assay. That may be due to the withdrawal of adriamycin from the cell culture medium for a relatively long time[29].

The general obstacle to cancer gene therapy is the delivery of a therapeutic gene. Additional problems of clinical P-gp inhibition by anti-MDR1 siRNAs may arise from the physiological expression of this ABC transporter in several epithelial and endothelial cells. For example, P-gp is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain[30], suggesting that this efflux pump plays an important role in the blood-brain barrier and is crucial for limiting the potential neuro-toxicity of many anticancer drugs. Thus, in clinical practice the application of P-gp-inhibiting shRNA expression vectors should be restricted to P-gp expressing cancer cells. One promising strategy for cancer cell-specific delivery of siRNAs targeting MDR1 is to develop vector systems delivering siRNA in a cell-type specific manner. Lentiviral vectors could offer some advantages in upcoming preclinical studies[31]. A "replication-defective" E1A-mutant adenoviral vector that efficiently and selectively replicates in "classic" multidrug-resistant cells has been reported[32]. These viral vectors may contribute to developing more useful vectors for the MDR reversal gene therapy in multidrug-resistant cancers.

In conclusion, the highly adriamycin-resistant HepG2/ADM cells can be completely reversed to an adriamycin-sensitive phenotype by the shRNA-mediated RNAi. The target sequence can be integrated into gene therapy vectors for the potential application in clinic.

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VIRAL HEPATITIS

Matrix-derived serum markers in monitoring liver fibrosis in children with chronic hepatitis B treated with interferon alpha

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Abstract

AIM: To evaluate prospectively 4 selected serum fibrosis markers (tenascin, hyaluronan, collagen VI, TIMP-1) before, during and 12 mo after IFN treatment of children with chronic hepatitis B.

METHODS: Forty-seven consecutive patients with chronic hepatitis B (range 4-16 years, mean 8 years) underwent IFN treatment (3 MU tiw for 20 wk). Fibrosis stage and inflammation grade were assessed in a blinded fashion before and 12 mo after end of treatment. Serum fibrosis markers were determined using automated assays.

RESULTS: IFN treatment improved histological inflammation but did not change fibrosis in the whole group or in subgroups. Only hyaluronan correlated significantly with histological fibrosis(r = 0.3383, P = 0.021). Basal fibrosis markers did not differ between responders (42.5%) and nonresponders(57.5%). During IFN treatment only serum tenascin decreased significantly in the whole group and in nonresponders. When pretreatment values were compared to values 12 mo after therapy, TIMP-1 increased in all patients and in nonresponders, and hyaluronan decreased in all patients and in responders.

CONCLUSION: Tenascin reflects hepatic fibrogenesis and inflammation which decreases during IFN treatment of children with chronic hepatitis B. TIMP-1 correlates with nonresponse and hyaluronan with histological fibrosis.

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Key words: Biopsy; Collagen VI; Fibrogenesis; Fibrosis; Hepatitis B virus; Hyaluronan; Serum marker; Tenascin; TIMP-1

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INTRODUCTION

The potential of interferon α (IFNα) to normalize aminotransferase activity, eliminate serum HBV DNA and HBeAg and reduce liver necroinflammation in patients with chronic hepatitis B is widely acknowledged[1-3]. In children with chronic hepatitis B treatment with IFN leads to long-term serological and biochemical remission in less than 50%[4-6]. Nonetheless, several reports suggested that IFN treatment for hepatitic C can halt or even reverse liver fibrosis[7-10], while its antifibrogenic potential in chronic hepatitis B needs to be confirmed[11]. Histological staging of liver fibrosis plays a central role in the liver pathological assessment since progressive fibrosis may lead to cirrhosis, the most important predictor of decompensated liver diseases and death[3, 12].

Liver biopsy is the standard method to assess fibrosis stage which allows to semiquantify the extent of fibrosis, yielding a static view[13]. However, biopsy has significant disadvantages for the assessment of fibrosis and fibrosis progression. Thus despite minor histological fibrosis, progression might be fast, since the accumulated extracellular matrix is the result of a dynamic process characterized by changes in matrix synthesis (fibrogenesis) and removal (fibrolysis)[14]. Furthermore, liver biopsy is invasive and histological scoring systems are not sensitive enough to detect small changes in fibrosis stage. Finally, bioptical sampling error can reach 25%-33% for a difference in one stage, when using the METAVIR system which ranges from 0 (no fibrosis) to 4 (cirrhosis)[15,16]. Therefore, noninvasive markers that may reflect overall hepatic fibrogenesis and fibrolysis in chronic hepatitis would be of great clinical benefit,

allowing repeated assessment of progression or therapeutic interventions, especially in children with chronic hepatitis B or C who are treated with IFN or other antiviral or potential antifibrotic agents[17-19].

The aim of this study was to investigate the clinical usefulness of selected matrix-derived serum markers (tenascin, hyaluronan, collagen VI, tissue inhibitor of metalloproteinase 1 or TIMP-1) in a long-term follow-up of children with chronic hepatitis B treated with IFN α.

MATERIALS AND METHODS

Patients

The study was carried out prospectively in 47 children (mean age 8 years, range 4-16, 31 boys and 16 girls) with serologically and biopsy-verified chronic hepatitis B. The children were positive for HBs and HBe antigens and had increased serum activity of HBV DNA polymerase for at least 1 year. Patients with autoimmune hepatitis or HCV coinfection were excluded from the study. None of the children was treated with antiviral and immunomodulating drugs during the 12-month period before inclusion into the study. Informed consent was obtained from all patients' parents and the protocol was approved by the local ethical committee of the Medical University of Bialystok. Serum samples were evaluated at three time points: at the start and the end (5 mo) of IFN α treatment, and 12 mo after end of treatment. Serum samples were stored at -70 ℃ until use. Standard liver tests were measured by validated automated methods and included total bilirubin, albumin, alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (gamma-GT). HBsAg and HBeAg were determined by MEIA (IMx, Abbott).

IFN treatment and definition of response

IFN (IFN α 2a: 30 children or IFN α 2b: 17 children) was applied at the dose of 3 MU tiw subcutaneously for 20 wk according to the schedule approved by the Polish Interferon Study Group[20]. HBeAg/antiHBe seroconversion and lack of HBV DNA polymerase activity 1 year after end of treatment was considered as the criterion of treatment response.

Measurement of serum fibrosis markers

Monoclonal antibodies were used to detect tenascin, collagen VI and TIMP-1 in sandwich immunoassays performed in an automated analyzer employing fluoresceine-labelled capture antibodies and alkaline phosphatase labeled detection antibodies. Hyaluronan was determined using biotinylated cartilage link protein. The immune complexes were separated from serum using magnetic particles covered with monoclonal anti-fluoresceine (anti-biotin in case of hyaluronan). The assays were developed for the BAYER IMMUNO 1 immunoassay system and validated in several cohorts of liver patients and healthy individuals[21-23].

Histological analysis

Percutaneous liver biopsies were obtained before treatment and 12 mo after IFN α discontinuation. The liver specimens were fixed in buffered formalin and embedded in paraffin. Histological sections were stained using hematoxylin-eosin, Masson-Goldner, Masson's trichrome and reticulin stains. Fibrosis stage and inflammation grade were assessed in a blinded fashion by a single pathologist according to the method of Batts and Ludwig[24].

Table 1 Baseline characteristics of the group of children with chronic hepatitis B

Data of the patients	Mean	SD	Minimum	Maximum
Age (yr)	8	3.51	4	16
HBV infection (mo)	44	30	10	144
ALT (nkat/L)	1600	1567	350	9902
AST (nkat/L)	1367	1117	567	7718
GGT (nkat/L)	250	150	50	1150
Bilirubin (μmol/L)	8.5	4.3	3.4	23.9
Albumin (g/L)	65.6	4.2	57.2	74.3
Hyaluronan (μg/L)	34.3	21.5	14.6	113.5
Tenascin (μg/L)	748.8	265.9	295.6	1480.2
TIMP-1(μg/L)	558.1	121.2	342.2	852.3
Collagen VI (μg/L)	5.8	2.2	2.3	14.2
Staging	2.0	0.6	1	3
Grading	1.6	0.7	1	3

Normal ranges: AST: 167-667 nkat/L; ALT: 167-667 nkat/L; GGT: 150-583 nkat/L; Bilirubin: 1.7-18.8 μmol/L; Albumin: 58.8-69.6 g/L.

Statistical analysis

Results were expressed as means ± SD. Statistical analysis was performed with the Wilcoxon rank-sum test for independent samples and Wilcoxon signed rank test for paired samples. The relationship between the serum fibrosis markers and liver histology scores was analysed by the Spearman rank-correlation test for nonparametric data and by the Pearson method for parametric data. Tests were considered statistically significant at $P < 0.05$.

RESULTS

Patient characteristics

The baseline characteristics of the 47 children are presented in Table 1. They were classified into responders (n = 20; 42.5%) and nonresponders (n = 27; 57.5%). There were no significant differences between the groups regarding age, gender, body mass, duration of HBV infection, the levels of bilirubin and albumin, the activity of GGT, levels of baseline liver fibrosis markers (tenascin, hyaluronan, collagen VI, TIMP-1) and grade of inflammation. However, treatment responders displayed a significantly higher activity of ALT (2184 ± 2217 *vs* 1150 ± 550 nkat/L) and AST (1717 ± 1567 *vs* 1100 ± 500 nkat/L) (P = 0.0453 for both) and a higher fibrosis score according to Batts and Ludwig (2.3 ± 0.5 *vs* 1.8 ± 0.6; P = 0.004).

Basal levels of serum fibrosis markers

We arbitrarily defined mild/moderate fibrosis as stage ⩽ 2 (n = 39) and advanced fibrosis as stage = 3 (n = 8) accor-

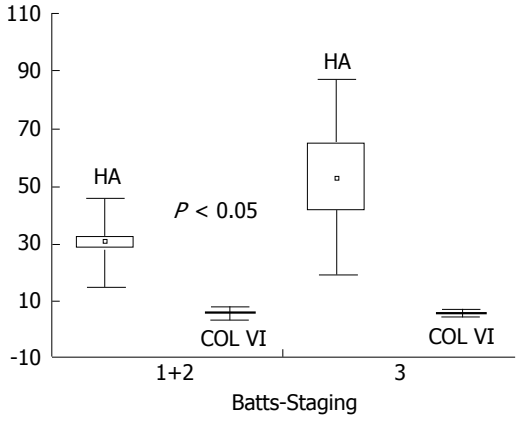


Figure 1 Correlation of serum fibrosis markers with histological staging (mean ± SD).

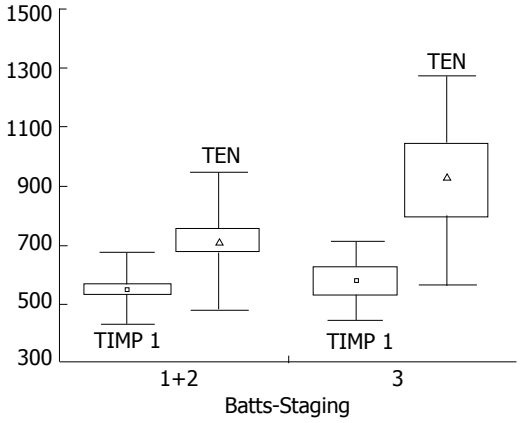


Figure 2 Correlation of serum fibrosis markers with histological staging (mean ± SD).

Table 2 Effect of IFN alpha on serum fibrosis markers in children with chronic hepatitis B (mean ± SD)

Patients	Marker (μg/L)	Before IFN (1)	After IFN (2)	12 mo after IFN (3)	*P* 1 *vs* 2	*P* 1 *vs* 3	*P* 2 *vs* 3
All	TIMP-1	558.1 ± 121.2	569.2 ± 134.8	636.9 ± 125.8	NS	b	NS
n = 47	CollagenVI	5.8 ± 2.2	6.2 ± 1.8	Nd	NS	-	-
	Tenascin	748.8 ± 266.0	641.5 ± 216.8	764.0 ± 250.0	b	NS	b
	Hyaluronan	34.3 ± 21.5	40.0 ± 30.9	28.7 ± 19.3	NS	b	b
Responders	TIMP-1	544.1 ± 120.3	555.4 ± 109.3	608.0 ± 134.9	NS	NS	NS
n = 20	CollagenVI	5.6 ± 1.85	6.7 ± 1.8	Nd	NS	-	-
	Tenascin	766.2 ± 289.9	686.2 ± 225.8	765.5 ± 249.9	NS	NS	NS
	Hyaluronan	40.3 ± 28.1	36.8 ± 18.0	30.7 ± 17.2	NS	a	a
Non	TIMP-1	567.9 ± 123.1	581.2 ± 155.0	657.2 ± 118.3	NS	b	NS
Responders	CollagenVI	6.0 ± 2.4	5.7 ± 1.7	Nd	NS	-	-
n = 27	Tenascin	736.6 ± 252.7	602.6 ± 205.6	762.9 ± 254.9	b	NS	b
	Hyaluronan	29.6 ± 13.6	42.7 ± 39.1	27.2 ± 20.9	NS	NS	b

Nd: Not determined; NS: Not significant; $^{a}P < 0.05$, $^{b}P < 0.01$.

ding to Batts and Ludwig. There were no significant differences in mean serum levels of tenascin, collagen VI and TIMP-1 between children with mild and advanced liver fibrosis, while levels of hyaluronan were higher in the latter group (30.3±15.8 *vs* 53.1±34.3 μg/L; $P = 0.0266$). There was a trend for increased tenascin and TIMP-1 in children with advanced fibrosis (Figure 1, Figure 2). We also arbitrarily defined mild inflammation as grade 1 ($n = 20$) and severe inflammation as grade ⩾2 ($n = 27$) according to Batts and Ludwig. There were no significant differences in mean concentrations of all serum fibrosis markers between children with mild and severe hepatic inflammation.

Effect of IFN on serum fibrosis markers

At end of treatment there were no significant changes in serum fibrosis markers in responders, while in nonresponders only tenascin decreased significantly ($P = 0.0074$). Twelve months after end of treatment serum hyaluronan was significantly lower than before treatment ($P = 0.0076$), while serum TIMP-1 was increased ($P = 0.0072$). In responders only hyaluronan decreased significantly ($P = 0.0304$), while in nonresponders the level of TIMP-1 increased ($P = 0.0064$, Table 2). Tenascin reached pretreatment levels in both responders and nonresponders.

Effect of IFN on liver histology

There were no significant changes in fibrosis stage after IFN therapy in the whole cohort, 2.0 ± 0.6 *vs* 2.1 ± 0.6, according to Batts and Ludwig and in subgroups, responders: 2.3 ± 0.5 *vs* 2.2 ± 0.7; nonresponders: 1.8 ± 0.6 *vs* 2.0 ± 0.6. Histological inflammation improved significantly in the whole group, 1.6 ± 0.7 *vs* 1.2 ± 0.7, $P = 0.0373$.

Correlation between serum fibrosis markers, histology and biochemical parameters

There were no significant correlations between baseline levels of the 4 serum fibrosis markers with liver fibrosis or inflammation according to Batts and Ludwig, or with AST, ALT, GGT, albumin or bilirubin. Only hyaluronan correlated significantly with histological fibrosis ($r = 0.3383$, $P = 0.021$).

DISCUSSION

Liver biopsy has been considered the gold standard for the assessment of hepatic fibrosis. Current recommen-

dations suggest that this procedure precede antiviral treatment in most patients with chronic hepatitits B or C[25]. However, liver biopsy is invasive with the potential for complications, such as bleeding which occurrence ranges from 0.3% to 0.5%[26,27], and mortality up to 0.1%[26,28]. In addition, since the biopsy core only represents 1/20 000 to 1/50 000 of the liver, biopsy is prone to sampling error, and variations in fibrosis staging may be high among different pathologists[15,16,21,29]. For these reasons, especially in children, non-invasive detection of histological liver damage, paticularly of fibrosis, is needed. Ideally serum markers of fibrosis should be applicable to patients with chronic hepatitis to either diagnose the stage of liver fibrosis, potentially replacing liver biopsy for this purpose, or to monitor progression of fibrosis or fibrogenesis, particulary during treatment [17,30]. Markers of the dynamics of fibrogenesis and fibrolysis are urgently needed, e.g. for short-term assessment of antifibrotic drug effects, but difficult to validate.

In this study we evaluated the changes of 4 serum fibrosis markers derived from the extracellular matrix (tenascin, hyaluronan, collagen VI and TIMP-1) before, at the end of and 12 mo after treatment of children with chronic hepatitis B with IFN. Our results showed a significant decrease of hyaluronan in responders and increased TIMP-1 in nonresponders, when levels before and 12 mo after interferon α treatment were compared. While falling during treatment, serum tenascin reached pretreatment levels in both responders and nonresponders. There were no significant changes in histological liver fibrosis 12 mo after the 5-mo course of IFN in all patients or in the subgroups of responders and nonresponders. This was expected, since the rate of fibrosis progression or regression in patients with chronic hepatitis B or C was usually slow. Assuming that IFN has at least some antifibrotic activity, as suggested before in large retrospective analyses of patients with chronic hepatitis C[31, 32], the histological scoring systems are obviously not sensitive enough to detect small changes in liver fibrosis and (modest) antifibrotic treatment effects. Nonetheless, the course of serum fibrosis (fibrogenesis) markers in our small but well defined group of children suggests that IFN indeed has antifibrogenic activity, especially in responders. This antifibrotic effect seems to be transient, as exemplified by serum tenascin which was depressed only during IFN treatment.

Prior to our study there had been no longitudinal, prospective studies of serum fibrosis markers in children with chronic hepatitis B, and only few studies analysed the effect of IFN therapy on the stage of liver fibrosis in children. Our findings are consistent with our previous study[33] and with those of others, who did not observe improvement of liver fibrosis by antiviral treatment in children, when biopsy was performed before and immediately after[34] or 9-12 mo after end of treatment[35, 36], while Gregorio *et al*[37] found significant improvement in staging in responders. However, these reports included small numbers of patients (≤24). It has been demonstrated that fibrosis stage changes more slowly than inflammation grade[7, 38]. This explains why we did not observe a significant improvement in fibrosis, while inflammation was clearly suppressed by IFN treatment.

We found that hyaluronan was the best serum marker to predict advanced liver fibrosis, since its level correlated significantly with histological fibrosis and was significantly higher in children with advanced *vs* mild/moderate liver fibrosis. These data are in keeping with previous results in patients with chronic viral hepatitis[39-43]. Thus the ability of this test to differentiate patients with extensive liver fibrosis from those with mild liver fibrosis was stronger than that of other markers, i.e., PIIINP, collagen IV, MMP-1, MMP-2 and TIMP-1[44] and laminin, collagen IV, PIIINP and TGF β1[45].

In children, up to now serum hyaluronan had only been studied in biliary atresia and cystic fibrosis[46-48], and there had been no data on collagen VI in chronic viral hepatitis or on TIMP-1 or tenascin in childhood liver diseases in general. Previous studies indicated that most serum fibrosis markers are influenced by body growth, especially PIIINP[49,50]. Thus healthy children have higher PIIINP levels than adults, excluding its use as reliable fibrosis marker for pediatric patients. Hyaluronan appears to be a useful marker of fibrosis stage also in children due to its short biological half life of only a few minutes and a prominent uptake by sinusoidal endothelial cells[51]. Similarly, tenascin and TIMP-1 are applicable to children with chronic hepatitis B as markers of fibrogenesis/inflammation and of fibrogenesis, respectively.

Our data suggest that serum hyaluronan, tenascin and TIMP-1 could be useful fibrosis markers in future studies of children with chronic viral hepatitis.

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BASIC RESEARCH

Identification of biomarkers of human pancreatic adenocarcinomas by expression profiling and validation with gene expression analysis in endoscopic ultrasound-guided fine needle aspiration samples

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Abstract

AIM: To compare gene expression profiles of pancreatic adenocarcinoma tissue specimens, human pancreatic and colon adenocarcinoma and leukemia cell lines and normal pancreas samples in order to distinguish differentially expressed genes and to validate the differential expression of a subset of genes by quantitative real-time RT-PCR (RT-QPCR) in endoscopic ultrasound-guided fine needle aspiration (EUS-guided FNA) specimens.

METHODS: Commercially dedicated cancer cDNA macroarrays (Atlas Human Cancer 1.2) containing 1176 genes were used. Different statistical approaches (hierarchical clustering, principal component analysis (PCA) and SAM) were used to analyze the expression data. RT-QPCR and immunohistochemical studies were used for validation of results.

RESULTS: RT-QPCR validated the increased expression of *LCN2* (lipocalin 2) and for the first time *PLAT* (tissue-type plasminogen activator or *tPA*) in malignant pancreas as compared with normal pancreas. Immunohistochemical analysis confirmed the increased expression of *LCN2* protein localized in epithelial cells of ducts invaded by carcinoma. The analysis of *PLAT* and *LCN2* transcripts in 12 samples obtained through EUS-guided FNA from patients with pancreatic adenocarcinoma showed significantly increased expression levels in comparison with those found in normal tissues, indicating that a sufficient amount of high quality RNA can be obtained with this technique.

CONCLUSION: Expression profiling is a useful method to identify biomarkers and potential target genes. Molecular analysis of EUS-guided FNA samples in pancreatic cancer appears as a valuable strategy for the diagnosis of pancreatic adenocarcinomas.

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Key words: Pancreas; Colon; Adenocarcinoma; Gene expression profiling; Endoscopic ultrasonography; Ultrasound; Fine needle aspiration

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INTRODUCTION

Pancreatic cancer remains one of the most deadly tumor types. The 5-year survival rate after diagnosis is less than 3.5%[1]. So far, the difficulties persist to diagnose pancreatic cancer early. Indeed, the molecular mechanisms underlying pancreatic oncogenesis remain partially understood.

Several fundamental studies suggest the implication of a number of molecules involved in cell cycling, apoptosis or signal transduction. Unfortunately, the clinical relevance of these molecules is still pending. For example, the screening of the activating mutations of the proto-oncogene *K-ras*, which are the most frequent alterations observed to date in pancreatic cancer, is not thought to be sufficiently robust as a diagnostic or prognostic marker[2]. The combination of several genetic alterations such as those found in tumor suppressor genes *p16, DPC4, p53* did not improve the sensitivity and specificity of K-*ras* mutation test for the diagnosis[3]. The development of new diagnostic tools is hence crucial for the detection of pancreatic cancer at an early stage.

Large-scale analysis of gene expression has been widely proposed as a powerful method for malignancy diagnosis, predicting invasion and metastasis through the identification of biomarkers. Pancreatic cancer has previously been the focus for such studies[4-8]. However, a rather low concordance between different studies was found in a meta-analysis of several of these studies[9]. There are several possible explanations; one of them being differences in probe design (oligonucleotides or cDNA), support (nylon membranes or glass slides) or the underlying detection technology (fluorescence or radioactivity). No matter what the reason is, there are controversies about the need of multiple well-defined and validated approaches to find a transcriptional "consensus" for a given tissue or cell type.

In this study, we used commercially dedicated macroarrays containing 1176 genes, selected on their functional implication in cancer biology, to study the expression profiles in pancreatic adenocarcinomas (surgical specimens and cell lines). We compared these profiles with those of normal pancreas, other adenocarcinoma cell lines of colon origin as well as with a non-adenocarcinoma leukemia cancer cell line. This array has never been used in expression profiling studies of pancreatic adenocarcinomas.

An important issue is the limited access to pancreatic tissue specimens. Being a minimally invasive technique for patient exploration, endoscopic ultrasound-guided fine needle aspiration (EUS-guided FNA) is now largely used for pancreatic tumor diagnosis[10]. Therefore, to support the clinical relevance of these studies, we determined whether quantification of these markers would be feasible in EUS-guided FNA specimens for prognostic or molecular diagnosis procedures.

MATERIALS AND METHODS

Cell and tissue samples

All cell lines were of human origin and were grown at 37℃, 50 mL/L CO_2 in the presence of 10 mL/L fetal calf serum, penicillin/streptomycin (Invitrogen Inc. Carlsbad, CA, USA) and 2 mmol/L L-glutamine (Invitrogen). ASPC-1 (ATCC: CRL-1682), Capan-1 (ATCC: HTB-79), Capan-2 (ATCC: HTB-80), NP29 (kindly provided by Dr. Gabriel Capellá, Barcelona, Spain), HCT 116 (ATCC: CCL-247) and K562 (ATCC: CLL-243) cells were grown in RPMI 1640 medium (Invitrogen). PANC-1 (ATCC: CRL-1469), B × PC-3 (ATCC: CRL-1687), SW480 (ATCC: CLL-228), SW620 (ATCC: CLL-227) and MIAPaCa-2 (ATCC: CRL-1420) cells were grown in Dulbecco's modified Eagle medium (DMEM), 1.0 g/L glucose. HT-29 cells (ATCC: HTB-38) were maintained in DMEM with 4.5 g/L glucose. Caco-2 cells (ATCC: HTB-37) were grown in RPMI 1640 in the presence of 10 g/L non-essential amino acids. Pancreatic ductal adenocarcinoma specimens were obtained from patients undergoing pancreaticoduodenectomy, after written consent and in accordance with French ethical guidelines. Pancreatic cancer samples were obtained through EUS-guided FNA in patients who have given their written consent. The protocol was approved by the Ethical Committee from Midi-Pyrénées CPPRB-1. Briefly, FNA was performed using GF-UC 30p ultrasound endoscope (Olympus, Rungis, France). Samples of pancreatic cancer tissue were obtained from each patient. The core biopsies were then transferred in Dubosq-Brazil medium and the cellular material remaining in the needle was immediately put in RNA later (Ambion, Woodward Austin, TX, USA). All cases of pancreatic cancer were diagnosed based on histological features. One of the normal pancreatic specimens was obtained from an organ donor. The other two normal pancreatic RNA samples were from Clontech (Palo Alto, CA, USA), each a pool from two individuals.

RNA extraction, cDNA labeling and membrane hybridization

Cells were rinsed in PBS and total RNA was extracted with the RNeasy mini-kit (QIAGEN, Valencia CA, USA). Adenocarcinoma tissues were first grinded mechanically in liquid nitrogen with a pestle in a mortar. Cellular samples obtained by EUS-guided FNA were temporarily stored at -25℃ in RNA later (Ambion), and total RNA purified using the RNeasy micro-kit (QIAGEN). The quality and the quantity of the RNA were systematically determined with an Agilent Bioanalyzer 2100. Atlas Pure Total RNA Labeling System (Clontech) and α^{33}P-dATP (-92.5 TBq/mmol) (GE-Amersham, Saclay, France) were used for cDNA target synthesis of 25-40 μg of total RNA. Only targets with a total activity superior to 1 million cpm were used. The membranes (Atlas Human Cancer 1.2, Clontech) were hybridized overnight, washed according to the manufacturer's recommendations and exposed on phosphor storage screens for generally three days. The screens were then scanned in a phosphorimager. All cell lines were analyzed at least twice, i.e. independent RNA extraction, labeling and hybridization procedures.

Expression data analysis

The scanned images were analyzed with the ImaGene software (V.4.0) (Biodiscovery Inc. Los Angeles, CA). Spots having an intensity inferior to the negative spots (foreign DNA) on the membrane (mean + 2SD) in more than 50% of the experiments in each category were eliminated. By this procedure, 871 genes were retained and used for further analysis. The gene names used are respecting the nomenclature proposed by HUGO. Functions and tissue expression distribution came from the database SOURCE available at http://source.stanford.edu or from

Table 1 Sense (S) and antisense (AS) primers used in quantitative RT-PCR analyses

Primer	Exon	Sequence (5′-3′)	Amplicon
LCN2 (Lipocalin2) S	1	TGATCCCAGCCCCACCT	74 bp
LCN2 (Lipocalin2) AS	2	CCACTTCCCCTGAATTGGT	
PLAT (tPA) S	2	TGGAGAGAAAACCTCTGCGAG	72 bp
PLAT (tPA) AS	3	CCATGATTGCTTCACAGCGT	
KRT7 (Keratin7) S	7	CTCTGTGATGAATTCCACTGGTG	72 bp
KRT7 (Keratin7) AS	8	CCCATGGTTCCCCCGA	
18S S		AAACGGCTACCACATCCAAG	155 bp
18S AS		CCTCCAATGGATCCTCGTTA	

the relevant references as indicated. The Genesis software (V1.5.0)[11] was used for clustering analysis. For principal component analysis (PCA) with biplot representation [12] the R software (V.1.9) was used (http://www.r-project.org/). FDR analysis was performed with the Significance Analysis of Microarrays (SAM) software (V1.21): http://www-stat.stanford.edu/~tibs/SAM/.

Real-time RT-QPCR

For quantitative real-time RT-PCR (RT-QPCR) analysis, 3 μg (tumor samples, cell lines, and normal pancreas) or 5-10 ng (cell aspiration) of total RNA were reversely transcribed using Superscript II RNase H$^-$ reverse transcriptase (Invitrogen) and random hexamers using standard conditions. Generally, 5% of the RT reaction was used as template for the subsequent RT-QPCR reactions using the SYBR Green technology (Applied Biosystems Foster City, CA, USA) in a GeneAmp 5700 sequence detector system (Applied Biosystems). The expression levels were normalized to 18S ribosomal RNA levels. The sequence, orientation and corresponding exon localization of the primers used are shown in Table 1 as well as the length of the amplicon.

Immunohistochemical analysis

Paraffin-embedded tissue sections were dewaxed and permeabilized with citrate buffer, pH 6, for 3 min ×5 min in a microwave oven. The slides were rinsed 3 min× 5 min in PBS at room temperature and treated for 10 min with Dako protein block solution (Dako, Glostrup, Denmark). The sections were then incubated with a monoclonal mouse anti-human lipocalin 2 antibody (clone 211-01) (The Antibody Shop, Denmark), (1:100 in PBS, 10 g/L BSA) over night at 4℃. After rinsing (3 min × 5 min in PBS), the sections were treated with 30 mL/L H_2O_2, 100 mL/L methanol in PBS for 15 min followed by two rinses (2 min × 5 min) in PBS, 10 g/L BSA, and the incubation with an HRP-coupled rabbit anti-mouse IgG antibody (P0161; Dako) (1:50 in PBS, 10 g/L BSA) for 1 h at 20℃. The slides were then rinsed 3 min × 5 min in PBS + 10 g/L BSA and incubated 1 h (20℃) with an HRP-coupled goat anti-rabbit IgG antibody (P0448; Dako) (1:50 in PBS, 10 g/L BSA) and finally rinsed 2 min × 5 min in PBS + 1% BSA prior to development with one drop of AEC + substrate chromogen solution (Dako) for 15 min. The slides were washed in running cold water for 10 min and counterstained with Mayer's hemalun solution (Merck, Darmstadt, Germany) and mounted with Dako glycerol mounting medium. Pictures were taken with the Visiolab 2000 software using a Nikon eclipse E400 microscope.

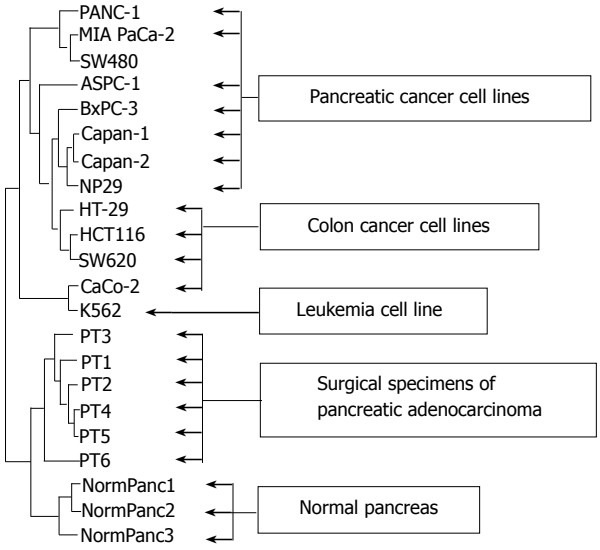


Figure 1 Hierarchical clustering analysis of sample expression profiles. Dendrogram of centered mean expression data using euclidian distance with average linkage clustering, showing relationships in gene expression profiles of samples. Closely related samples are found in the same branch of the tree and a reduced branch height represents a closer relationship between groups.

RESULTS

Expression profiling

Using Clontech's Human Cancer 1.2 macroarrays, the expression profile of 1176 genes associated with cancer was studied in 7 human pancreatic adenocarcinoma cell lines (PCL), PANC-1, MIA Paca-2, ASPC-1, BxPC-3, Capan-1, Capan-2, NP29, and 6 tumor samples, PT1-PT6, obtained through surgical resection of pancreatic adenocarcinomas. In order to assess the specificity of the expression of pancreatic tumor cells, 5 human colon cancer cell lines (CCL), SW480, HT-29, HCT116, SW620, Caco-2, 1 leukemia cell line (LCL), K562 and 3 normal human pancreatic tissue samples, NormPanc1-3, were included in the study. After screening of the expression data as described in the material and methods section, 871 genes were retained for further analysis. The expression data are available at the following URL: http://ifr31w3.toulouse.inserm.fr/microArrayPancreas/DataSummary.xls. In a hierarchical cluster analysis of samples based on centered mean expression data, two main clusters could be identified, corresponding to cell lines (up) and tissue samples (down), respectively (Figure 1). Among the clusters identified within the cell lines, one was purely pancreatic (BxPC-3, Capan-1, Capan-2 and NP29) and one comprised only colon cancer cell lines (HT-29, HCT116 and SW620). At the top of the dendrogram, a colon cancer cell line SW480 clustered with two pancreatic cancer cell lines, PANC-1 and MIA PaCa-2. In a hierarchical clustering analysis of the 871

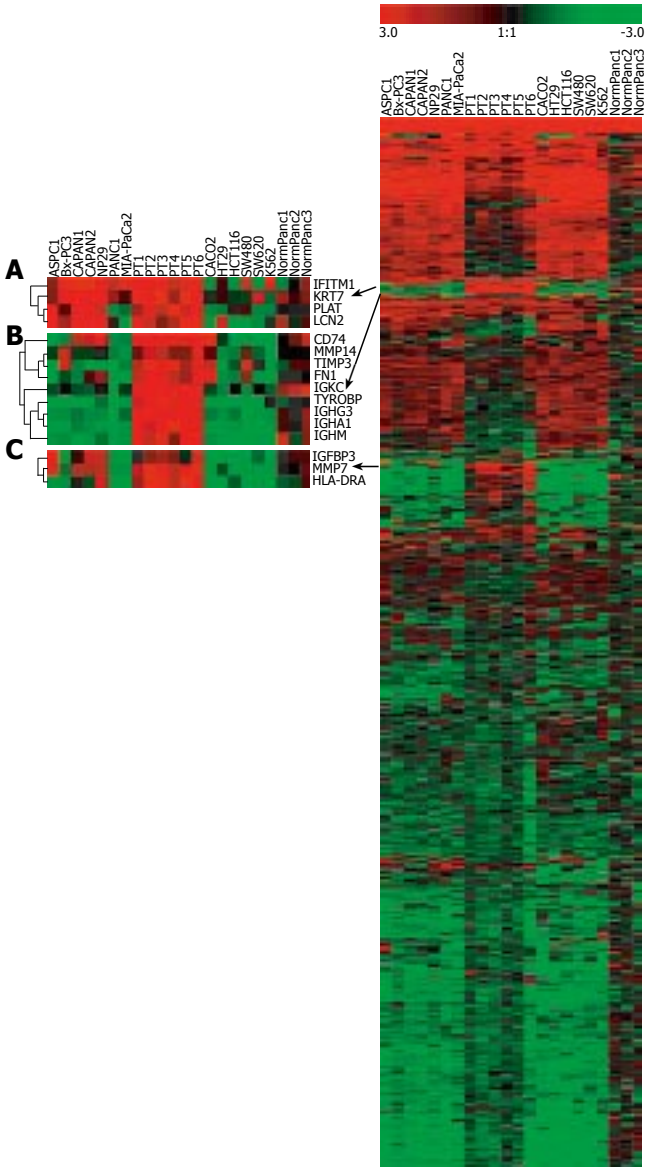


Figure 2 Hierarchical clustering analysis of gene expression profiles. Right: Expression profile of the 871 genes retained after data-filtering. Left: Three magnified subclusters (**A**-**C**) showing the genes overexpressed in most of pancreatic tumors. Red color indicates high expression levels; green color indicates low expression levels.

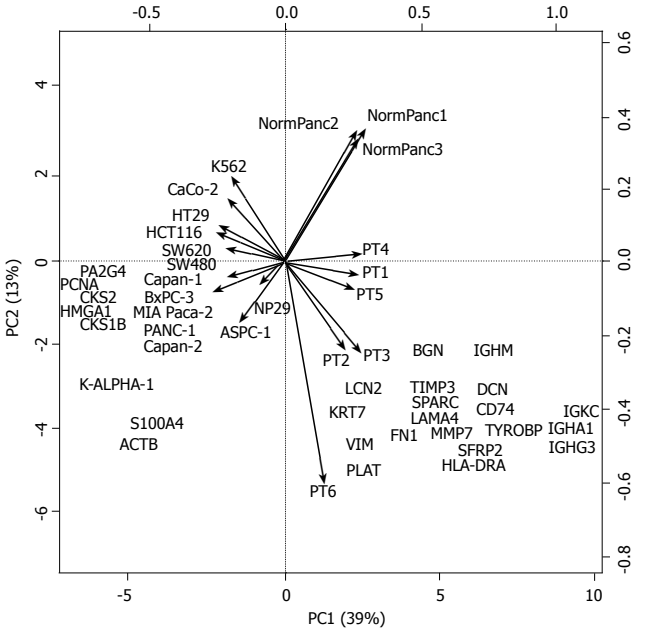


Figure 3 Principal component analysis (PCA) of gene expression data. Biplot resulting from a PCA of the line and column centered data containing 871 genes (individuals) in lines and 22 samples in columns (variables). The 28 genes contributing the most to the total variability are shown. The two principal components (PC1 and PC2) contribute to more than 50% (39% and 13%) of the total variability and resolve four biological sample categories: PC1 on the horizontal x-axis distinguish between cell lines (left) and tissue samples (right) whereas PC2 on the vertical y-axis distinguish between malignant pancreas samples (bottom) and other sample categories (top).

genes retained after filtering, three subclusters (A, B and C) contained genes with an elevated expression in pancreatic tumor samples (PT) and a lower expression in normal pancreas and non-pancreatic cancer cell lines (NormPanc, CCL and LCL) (Figure 2). Subcluster A containing four genes, *IFITM1*, *KRT7*, *PLAT* and *LCN2*, exhibited an expression profile particularly specific for neoplastic pancreas. These four genes were overexpressed in the majority of PCL and in PT1 to 6, but not in non-pancreatic cell lines and in normal pancreas. Subclusters B and C showed genes overexpressed in all PT, in some pancreatic cancer cell lines but not in normal pancreas.

In order to estimate the variability of the experimental procedures, several biological and technical replicates were performed. In a hierarchical clustering analysis, these replicates either clustered directly together or regrouped within clusters of the respective cell line validating our experimental protocols and data acquisition procedures (data not shown). We used the SAM software to detect the genes that were significantly over-expressed in malignant pancreas, referring to the mean expression in pancreatic tumors (PT) and pancreatic cancer cell lines (PCL) as compared with the other groups normal pancreas (NormPanc), colon cancer cell lines (CCL) and leukemia cell line (LCL). Among the genes selected by SAM (FDR < 2%), the ones with an at least 2-fold higher expression level in malignant pancreas are shown in Table 2. In the same table are also represented other expression fold ratios, i.e. the comparison between pancreatic and colon cancer cell lines (column PCL/CCL) as well as the fold ratio between pancreatic tumors and normal pancreas (column PT/NormPanc). The five genes with the highest expression ratios were *PLAT*, *KRT7*, *CD74*, *MMP7*, and *LCN2*. The ratio of mean expression level of these genes in PT as compared with that in NormPanc, was found to be equal to or above 3.36, indicating that they discriminate well between adenocarcinoma and normal tissues. The ratio of the gene mean expression level in PCL as compared with that in CCL discriminates between pancreatic and colon cancer cells, which is important to differential diagnosis.

Although clustering methods are the most widely used technique to group genes based on expression patterns, PCA is rapidly gaining acceptance in the field of transcriptome analysis. PCA permits data visualization of complex multidimensional datasets by projecting data into a sub-space with 2 or 3 dimensions. Moreover, a biplot representation of PCA results (Figure 3) provides

Table 2 Significantly overexpressed genes (over 2-fold) in malignant pancreas

Gene symbol	Gene description	Mean fold expression ratios		
		Malignant Panc/others	PCL/ CCL	PT/ NormPanc
PLAT	Plasminogen activator, tissue-type	6.22	4.36	6.74
KRT7	Keratin 7	5.72	4.92	3.97
CD74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	5.44	1.61	8.00
MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	5.36	3.73	5.70
LCN2	Lipocalin 2 (oncogene 24p3), NGAL	5.25	8.32	3.36
HLA-G	HLA-G histocompatibility antigen, class I, G	4.04	1.57	4.32
IGHG3	Immunoglobulin heavy constant gamma 3	3.95	0.94	11.81
HLA-DRA	Major histocompatibility complex, class II, DR alpha	3.70	1.57	7.01
TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	3.50	1.61	9.20
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	3.48	3.00	2.40
ITGB4	Integrin, beta 4	3.36	1.16	4.34
KRT19	Keratin 19	3.27	1.34	2.98
CTSD	Cathepsin D (lysosomal aspartyl protease)	3.21	2.75	2.89
CASP4	Caspase 4, apoptosis-related cysteine protease	3.10	2.43	1.80
IGFBP3	Insulin-like growth factor binding protein 3	3.08	3.53	1.87
PLAU	Plasminogen activator, urokinase	3.08	2.83	2.01
MMP11	Matrix metalloproteinase 11 (stromelysin 3)	2.96	1.11	7.22
DTR	Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	2.84	1.74	3.33
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.76	1.65	3.17
LAMA4	Laminin, alpha 4	2.74	2.14	3.30
ITGB8	Integrin, beta 8	2.59	2.31	4.44
IFITM1	Interferon induced transmembrane protein 1 (9-27)	2.41	5.47	7.10
AXL	AXL receptor tyrosine kinase	2.35	1.63	2.91
ITGAE	Integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	2.31	1.58	3.56
CD59	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344)	2.26	2.11	2.95
KRT10	Keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	2.09	2.18	1.60
CYR61	Cysteine-rich, angiogenic inducer, 61	2.03	2.41	1.22
PTGES	Prostaglandin E synthase	2.03	2.84	1.11
HIF1A	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	2.01	1.24	2.87

a powerful visual overview of the relationships between genes and samples. The sub-space determined by PCA captures the highest amount of the total variability. In our line and column centered data matrix, the first (PC1) and second (PC2) principal components captured 39% and 13% of the total variability, respectively (Figure 3). In the biplot, samples are shown as arrows, and the genes are shown by their HUGO ID symbols. Arrows of similar length, pointing in the same direction display a higher degree of correlation. As observed in the hierarchical clustering analysis (Figure 1), SW480 was more associated to pancreatic cell lines than to other colon cell lines. Also in agreement with figure 1, the leukemia cell line K562 was correlated with the colon cell line Caco-2. An analysis of the PC1 (horizontal x-axis) revealed a clear distinction between tissue samples and cell lines, with a positive x-coordinate value for all tissue samples and negative for all cell lines. More interestingly, the same analysis of PC2 (vertical y-axis) discriminated malignant pancreas (pancreatic tumors and pancreatic cell lines) from the other samples categories studied, with the exception of SW480.

To improve the interpretation, only the 28 genes contributing most to the total variability of the data set are shown in the biplot. The co-localization between a gene and a sample type signifies an overexpression in the given sample type. Accordingly, genes such as *PCNA*, *HMGA1*, *PA2G4*, *CKS2* and *CKS1B* were specifically overexpressed in cell line samples, whereas *IGHM*, *IGKC*, *IGHA1* and *IGHG3*, all immunoglobulins, were highly associated with tumor samples. The extreme position of these genes on the horizontal axis in the biplot indicates they are involved in the distinction between cells and tissue samples. The overexpression of genes of the immune response is a characteristic of cancer tissue samples versus cell lines. Furthermore, a set of genes, including *PLAT*, *VIM*, *HLA-DRA*, *IGHG3*, *ACTB*, *S100A4*, and others located in the lower half of the biplot, was involved in the discrimination between malignant pancreas and all other samples. Among them, *LCN2*, *KRT7*, *VIM* and *PLAT*, appeared to be highly correlated with both pancreatic tumors and pancreatic cell lines. However, in the third principal component (PC3), *VIM* was positioned far from the three other genes (data not shown). Interestingly, *CTRL* (chymotrypsin-like), was found to be specifically expressed in normal pancreas and absent in PT or PCL. CTRL is a poorly characterized serine protease with chymotrypsin- and elastase-2-like activities, which is expressed as a pro-protease in normal pancreatic tissue[13]. The absence of *CTRL* expression in adenocarcinoma samples is in agreement with the absence of other serine

Table 3 Relative mRNA levels presented as $2^{[Ct(18S)-Ct(gene\ of\ interest)]}$ (mean x 10^{-2} ± SEM)

Gene	*n*	KRT7	LCN2	PLAT
Pancreatic tumors	6	1.14. ± 0.47	3.58 ± 0.65[a]	2.34 ± 0.89[a]
Pancreatic cell lines	6	79.62 ± 90.97	188.99 ± 81.13[a]	13.51 ± 4.67[a]
Normal pancreas	3	0.42 ± 0.23	1.05 ± 0.85	0.14 ± 0.08

[a]$P < 0.05$, *vs* normal pancreas.

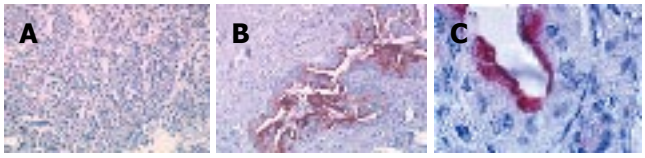


Figure 4 Lipocalin 2 protein expression in pancreatic cancer tissue Immunohistochemical analysis by AEC + substrate chromogen staining. **A** (x 40): normal pancreas; **B** (x 200), **C** (x 1000): pancreatic adenocarcinomas.

protease digestive enzyme, such as chymotrypsin and trypsin, present in normal pancreatic tissue and known to be down-regulated in pancreatic cancer.

Differentially expressed genes in neoplastic pancreas

Taken together, the three statistical methods used in our study (hierarchical clustering, Figure 2, the SAM method, Table 2, and PCA, Figure 3) retained seven genes as differentially overexpressed in malignant pancreas. Among these genes, 3 are involved in the immune response: *CD74*, *HLADRA*, and *IGHG3*, and 4 genes previously suggested as key proteins in pancreatic cell biology or oncogenesis**:** *KRT7*, *MMP7*, and *PLAT* and *LCN2*. Among them, three genes *LCN2*, *KRT7* and *PLAT* were selected for further validation by real-time RT-QPCR. This choice was based on the highly specific expression profile in malignant pancreas (Figure 2; subcluster A) and elevated mRNA levels in the corresponding samples (Table 2) as well as on their specific positioning in the PCA analysis, that is in the interface between the pancreatic tumors and pancreatic cancer cell lines. These studies confirmed significantly elevated levels of *LCN2* and *PLAT* mRNA in malignant pancreas samples, tumors and cancer cell lines (Mann-Whitney test: $^{a}P < 0.05$) (Table 3). However, they were unable to confirm the macroarray results concerning *KRT7* expression levels.

The PLAT protein is known to be overexpressed in pancreatic cancer tissues[14]. However, even though several studies have reported elevated *LCN2* mRNA levels in pancreatic cancer[15], and LCN2 protein in human pancreatic juice in patients with pancreatic cancer[16], the presence of the LCN2 protein in pancreatic adenocarcinoma is less well characterized. Therefore, in order to evaluate the expression of the lipocalin 2 protein in pancreatic tumors, we performed immunohistochemical analysis using a monoclonal anti-lipocalin 2 antibody. As shown in Figure 4A, little or no labeling was observed in normal pancreas ($n = 3$). However, in sections of pancreatic adenocarcinomas ($n = 5$), a strong apical labeling was detected in ducts invaded by carcinoma (Figure 4B and C).

LCN2, KRT7 and PLAT transcripts in EUS-guided FNA samples

We investigated the possibility to use EUS-guided FNA as a source of pancreatic sample to study the expression of *KRT7*, *LCN2* and *PLAT* genes as diagnostic biomarkers. The presence of these mRNAs was therefore examined by RT-QPCR analysis in 12 samples, originating from different individuals, obtained through EUS-guided FNA from patients with pancreatic adenocarcinoma. Compared with normal pancreas, the expression level of the *KRT7*, *LCN2* and *PLAT* transcripts were 26.7 ± 18.16, 20.6 ± 7.6 and 70.8 ± 35.4 (mean ± SE), respectively. In each case, 18S ribosomal RNA was used for normalization. Even though 10 out of 12 patients presented elevated levels of expression for *KRT7* and *LCN2* and 11 for *PLAT,* only the levels of *PLAT* and *LCN2* expression were significantly different from normal pancreas (unilateral Student *t* test: $P < 0.05$). These results were thus perfectly concordant with those obtained from pancreatic cancer cell lines and tumors.

DISCUSSION

Large scale analysis of gene expression has been widely proposed as a powerful method for identification of molecular markers of neoplasia and for the generation of novel taxonomies for cancer. Accordingly, several studies, have been conducted to characterize the expression profiles in human pancreatic adenocarcinomas[15, 17]. However, to the best of our knowledge, none of these studies report the comparison between pancreatic and colon cancer cell lines. Moreover, this study reports, for the first time, about the use of the dedicated Atlas Human Cancer 1.2 cDNA macroarray containing 1176 cancer-associated genes to study expression profile in pancreatic carcinoma. We report the expression profiles of pancreatic adenocarcinoma cell lines and tumors and compared with that of normal pancreas specimens, colon cancer cell lines and one hematopoietic cell line K562. This was done to differentiate pancreatic and non-pancreatic cancer gene profile in view of differential diagnosis. The fact that several groups have previously reported elevated expression levels of *LCN2* mRNA in pancreatic cancer[18-21], was reassuring for the validation of our study design. However, reports validating this overexpression at the protein level are very scarce[22]. Lipocalins are small extracellular proteins with important role in cell proliferation and differentiation, possessing protease inhibitory properties and/or carrying lipophilic ligands such as retinoids and fatty acids into the cells[23]. The localization of LCN2 protein at the apical side of cells from ducts invaded by the tumoral process might be of pathophysiological importance and supports further analysis for a better understanding of the role of this protein in cancer.

Interestingly, the overexpression of *PLAT* in pancreatic adenocarcinoma has not been reported in previous expression profiling studies. PLAT was recently shown to play a critical role in tumor angiogenesis and in the devel-

opment of exocrine pancreatic cancer[24], contributing to an invasive phenotype[14,25]. The vast majority of pancreatic adenocarcinomas express KRT7[26,27] and it has previously been indicated as overexpressed in pancreatic cancer by gene expression profiling studies[28]. However, the KRT7 protein is suggested as a marker of normal pancreatic duct epithelial cells playing a role in cell differentiation[29]. The reported expression of KRT7 in both normal and malignant pancreas could, at least in part, explain why we were unable to observe a significant difference in expression levels between both sample types. Among the seven genes retained on our study, three are highly implicated in the immune response: *CD74*, *HLADRA, IGHG3*. *IGHG3* was only overexpressed in tumor samples suggesting an immune cell origin. In contrast, elevated levels of *CD74* and *HLADRA* were found in tumor samples and also in some pancreatic cancer cell lines. Elevated levels of *CD74*, and *HLADRA* were observed in several types of cancers, including gastric cancer and renal epithelial neoplasms[30,31].

The quantitative RT-PCR on EUS-guided FNA samples of *PLAT* and *LCN2*, validated the overexpression found by the macroarray analysis. Thus, the quality and the amount of cellular sampling using pancreatic EUS-guided FNA allow the extraction of sufficient quantities of RNA to perform RT-QPCR analysis as a new tool for early diagnosis, as described recently for lymph node metastasis[32]. Furthermore, when performed directly on resected tumor pieces, fine needle aspiration has been shown to produce a relative enrichment of cancer cells, in comparison to tumor samples. This enrichment has been attributed to the capability of epithelial cancer cells to be aspirated more easily than stromal cells[33]. To the best of our knowledge, no gene expression had been evaluated with EUS-guided FNA biopsies from patients with pancreatic adenocarcinoma. EUS-guided FNA is a safe method for patient exploration. Morbidity rate ranges from 1% to 3% when performed by experienced endosonographers[34]. Therefore, identification and quantification of potential molecular markers for pancreatic cancer on cellular samples obtained by EUS-guided FNA could be a promising approach for the diagnosis of solid pancreatic masses. Future studies are needed to prospectively evaluate new molecular biomarkers using this procedure, in order to increase the accuracy of current standard histological and cytological analyses, that was only 80 to 85% in pilot studies[35,36].

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BASIC RESEARCH

Adiponectin deficiency exacerbates lipopolysaccharide/D-galactosamine-induced liver injury in mice

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Abstract

AIM: To examine the effects of adiponectin on the functions of Kupffer cells, key modulators of lipopolysaccharide (LPS) -induced liver injury.

METHODS: D-galactosamine (GalN) and LPS were injected intraperitoneally into adiponectin-/- mice and wild type mice. Kupffer cells, isolated from Sprague-Dawley rats, were preincubated with or without adiponectin, and then treated with LPS.

RESULTS: In knockout mice, GalN/LPS injection significantly lowered the survival rate, significantly raised the plasma levels of alanine transaminase and tumor necrosis factor-α (TNF-α) and significantly reduced IL-10 levels compared with wild type mice. TNF-α gene expression in the liver was which higher and those of IL-10 were lower in knockout mice than in wild type mice. In cultured adiponectin-pre-treated Kupffer cells, LPS significantly lowered TNF-α levels and raised IL-10 levels in the culture media and their respective gene expression levels, compared with Kupffer cells without adiponectin-pre-treatment.

CONCLUSION: Adiponectin supresses TNF-α production and induces IL-10 production by Kupffer cells in response to LPS stimulation, and a lack of adiponectin enhances LPS-induced liver injury.

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Key words: Adiponectin; Lipopolysaccharide; Kupffer cell; TNF-alpha; IL10

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INTRODUCTION

Obesity is currently a serious medical problem world wide. It is an independent risk factor of non-alcoholic steatohepatitis (NASH) and alcoholic liver injury[1,2]. The pathogenesis of these diseases involves lipopolysaccharide (LPS)[3-5]. The complex of LPS and LPS-binding protein (LBP) activates Kupffer cells to secrete tumor necrosis factor (TNF)-α, which plays an important role in liver injury[6,7]. The control of the response of Kupffer cells to LPS is thought to be critical in the prevention of LPS-induced liver injury. Indeed, the selective inhibition of Kupffer cells by the administration of either gadolinium chloride or methyl palminate results in the abrogation of liver injury with an inhibition in TNF-α secretion[8]. Interleukin (IL)-10, which is secreted by Kupffer cells in the liver after LPS stimulation[9], has strong anti-inflammatory effects in the liver, and prevents liver fibrosis[10,11]. Interferon (IFN)-γ activates the signal transducer and activator of transcription (STAT-1) in hepatocytes and promotes apoptosis in hepatocytes after administration of D-Galactosamine (GalN)/LPS[12]. In GalN/LPS induced liver injury, the modulation of the production of these cytokines from Kupffer cells is important.

Adiponectin, a 30 ku adipocyte complement-related protein (Acrp30), is an adipocyte-specific plasma protein (normal level, 5-30 mg/L), and is paradoxically decreased in obesity and in cases of alcoholic fatty liver[13,14]. Adiponectin contains two types of receptors, AdipoR1 and AdipoR2. Whereas AdipoR1 is expressed ubiquitously and abundantly in skeletal muscle, AdipoR2 is most abundantly expressed in the liver and is an intermediate-affinity receptor for full-length and globular adiponectin[15]. Adiponectin improves the insulin sensitivity, and has anti-

atherogenic and anti-inflammatory effects. In the liver, it has anti-fibrogenic effects and regulates hepatic stellate cells[16].

We recently reported that adiponectin inhibits the phagocytosis of human macrophages and LPS-induced TNF-α release[17], and induces IL-10 gene expression in human macrophages[18]. We hypothesized that adiponectin has anti-inflammatory effects on Kupffer cells, key modulators of LPS induced liver injury, by regulating the release of cytokines. However, the effects of adiponectin on LPS-induced liver injury and Kupffer cells remained poorly understood. In the present study, to clarify the effects of adiponectin on LPS-induced liver injury, we investigated the effects of adiponectin on GalN/LPS-induced liver injury using adiponectin knockout mice. We also examined the effects of adiponectin on cytokine production from Kupffer cells in primary cultures.

MATERIALS AND METHODS

All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine. Recombinant mouse full-length adiponectin was prepared as described previously[16]. The method of disruption of the mouse adiponectin gene was described previously[19].

Murine model of acute hepatitis

To clarify the role of adiponectin in LPS-induced liver injury, experiments were conducted using adiponectin-/- mice and wild-type (WT) control C57B6 mice (28-32 g body mass; 10-12 wk old)[19]. Each mouse was simultaneously injected intraperitoneally with 700 mg/kg of GalN and 10 μg/kg of LPS. The doses of GalN and LPS were determined in previous studies and our preliminary study[12]. LPS from *E. coli* O55:B5 and D-galactosamine (GalN) were purchased from Sigma (St. Louis, MO). Groups of 10 mice were treated to determine the survival curve. Groups of 6 mice were treated to measure the plasma levels of alanine aminotransferase (ALT), TNF-α, IL-10, and IFN-γ. Groups of 6 mice were sacrificed as follows: before, 0.5, 1, and 4 h after the administration to measure the gene expressions of TNF-α IL-10, IFN-γ in the whole liver and IFN-γ in the spleen, assessed by means of real-time polymerase chain reaction (PCR).

LPS stimulation on Kupffer cells

Male Sprague-Dawley (SD) rats (10 wk old, $n = 6$) were anesthetized with pentobarbital sodium, and the portal vein was cannulated. The liver was perfused with a ethylene glycol bis (beta-aminoethyl ether)-N, N, N', N' -tetraacetic acid (EGTA) solution, and digested with a 0.5 g/L collagenase solution. Differential centrifugation on Nycodenz (Pharma, Oslo, Norway) density gradients was performed as described before[20]. We evaluated the purity of the isolated Kupffer cell population by counting CD68 positive cells, ranging from 88.5% to 93.0%. Kupffer cells from each rat were maintained and treated separately. The Kupffer cells were maintained at 37°C under 50 mL/L CO_2 in Dulbecco's modified Eagle medium containing 100 mL/L fetal calf serum with or without adiponectin (10 mg/L) for 24 h. After a 24 h pretreatment with and without adiponectin, LPS was added to the culture medium at a concentration of 10 mg/L. The doses of adiponectin and LPS were determined in previous studies and our preliminary study[6,17]. TNF-α, IL-10, IFN-γ concentrations in the culture medium were measured 0 and 4 h after the commencement of LPS stimulation. Total RNA of Kupffer cells was isolated at 0, 0.5, 1, and 4 h after the commencement of LPS stimulation.

Measurement of plasma concentrations of ALT and cytokines

Plasma ALT concentrations were measured by using a transaminase CII-test kit according to the protocol provided by the manufacturer (Wako Pure Medical, Osaka, Japan). Circulating levels of TNF-α, IL-10, and IFN-γ were assessed using commercial enzyme-linked immunosorbent assay (ELISA) kits for mice (Biosource Int., Camarillo, CA). Their concentrations in the culture media of Kupffer cells were quantified using ELISA kits for the rat (Biosource Int.).

Quantification of gene expression levels

Total RNA from whole liver, spleen and Kupffer cells were extracted using a Qiagen (Hilden, Germany) QIAshredder and an RNA-easy Mini kit according to the instructions provided by the manufacturer. The reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously[21]. The Quantitect PCR probe kit and Quantitect gene assay kit for mice TNF-α, IL-10, and IFN-γ were purchased from Qiagen and used in a real-time PCR analysis of the mouse samples. Primers for rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH), TNF-α, IL-10, IFN-γ, and mice GAPDH were designed using the computer program Primer Express (Applied Biosystems, Foster city, CA). Dynamo SYBR Green qPCR kit (Finzymes, Espoo, Finland) was used for the real-time PCR analysis of rat TNF-α, IL-10, IFN-γ, GAPDH and mice GAPDH. Real-time PCR was performed using a DNA Engine Opticon 2 real-time PCR Detection System (MJ Research, Waltham, MA). Relative gene expression was quantified using GAPDH as an internal control. The expressions of adiponectin receptors, AdipoR1 and AdipoR2 were assessed with RT-PCR as previously published[22].

TUNEL assay

Quantification of apoptotic hepatocytes in liver sections was performed by counting the number of TUNEL-positive cells. The data were expressed as the average number of TUNEL-positive cells per five high power fields (HPF) (×100). The TUNEL assay was performed based on the instructions provided by the manufacturer (In Situ Apoptosis Detection Kit; TaKaRa, Shiga, Japan, and Liquid DAB Substrate kit; Zymed Lab. Inc., South San Francisco, CA).

Statistical analysis

The results are presented as the mean ± SE. Analysis of variance (ANOVA) for the groups was performed by the

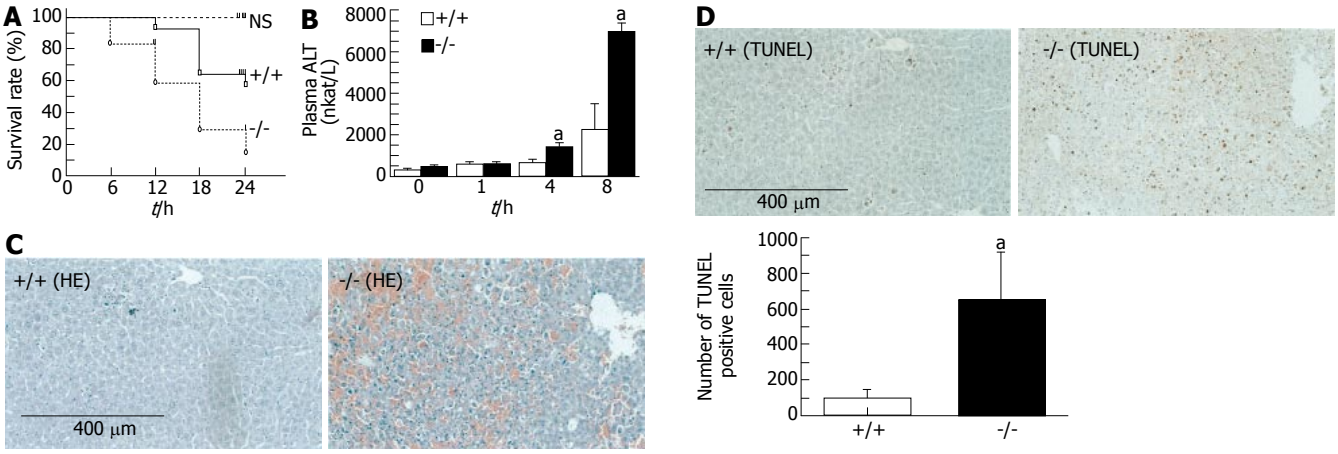


Figure 1 GalN/LPS induced changes in mice. **A:** Survival rate (n = 10); **B:** Plasma ALT (mean ± SE, n = 6). $^aP < 0.05$, $^bP < 0.01$, *vs* WT; **C:** Histology of the liver. Massive liver injury in adiponectin-/- mice. (Hematoxylin-eosin × 100); **D:** Large numbers of apoptotic hepatocytes in adiponectin-/- mice (TUNEL × 100). Scale bar = 400 μm. $^aP < 0.05$, *vs* WT mice.

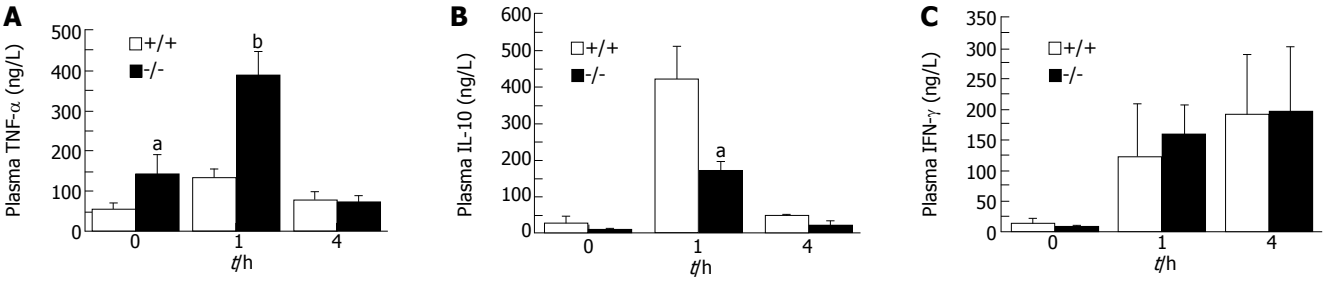


Figure 2 Plasma concentrations of TNF-α, IL-10 and IFN-γ in mice after GalN/LPS administration. (mean ± SE, n = 6). $^aP < 0.05$, $^bP < 0.01$, *vs* WT.

Mann Whitney test, followed by Scheffé's test for multiple comparisons to allow pairwise test for significant differences between groups. The statistical significance of the lethality rates was determined by a Log-rank test. Statistical significance was defined as $P < 0.05$.

RESULTS

GalN/LPS induces severe liver injury in KO mice

The survival rate of adiponectin-/- mice after GalN/LPS administration was significantly lower than that of WT mice 24 h after the administration ($P = 0.041$; log-lank test; Figure 1A). GalN/LPS administration resulted in an elevation of plasma ALT concentrations in both WT and adiponectin-/- mice, although increases at 4 and 8 h after administration were significantly higher in adiponectin-/- mice than in WT mouse (at 4 h; WT, 693.5 ± 472.8 *vs* adiponectin-/-, 1458.6 ± 541.8 nkat/L; $P < 0.01$, at 8 h; WT, 2255.5±3242.3 *vs* adiponectin-/-, 6988.1 ± 978.5; $P < 0.05$; Figure 1B). A histological examination revealed that GalN/LPS administration induced massive liver injury in adiponectin-/- mice mice (Figure 1C). The number of TUNEL-positive hepatocytes in adiponectin-/- mice livers was significantly higher than that in WT mouse livers 8 h after GalN/LPS administration (WT, 95 ± 118 *vs* adiponectin-/- mice, 683 ± 729 apoptotic cells/HPF; $P < 0.05$; Figure 1D).

Effects of GalN/LPS on plasma TNF-α, IL-10 and IFN-γ

Prior to GalN/LPS administration, plasma TNF-α concentrations were significantly higher in adiponectin-/- mice than in WT mice ($P < 0.05$; Figure 2A). Plasma TNF-α concentrations increased reaching peak levels 1 h after the administration, and were significantly higher in adiponectin-/- mice mice than in WT mice at their peak ($P < 0.01$; Figure 2A). Plasma IL-10 concentrations also increased, reaching peak levels 1 h after the administration of GalN/LPS. In adiponectin-/- mice, IL-10 plasma concentrations were significantly lower than those of WT mice at their peak ($P < 0.05$; Figure 2B). Plasma IFN-γ concentrations increased continuously up to 4 h after GalN/LPS administration, and no significant difference between adiponectin-/- mice and WT mice was found (Figure 2C).

Effects of GalN/LPS on liver TNF-α, IL-10, IFN-γ and splenic IFN-γ mRNA expression

The TNF-α mRNA expression level in the liver increased, reaching a peak level 1 h after GalN/LPS administration, and this level was significantly higher in adiponectin-/- mice than in WT mice 1 h after the administration ($P < 0.01$; Figure 3A). The IL-10 mRNA expression level in the liver also increased, reaching a peak level 4 h after GalN/LPS administration, and the levels at 1 and 4 h were significantly higher in WT mice than in adiponectin-/- mice (at 1 h;

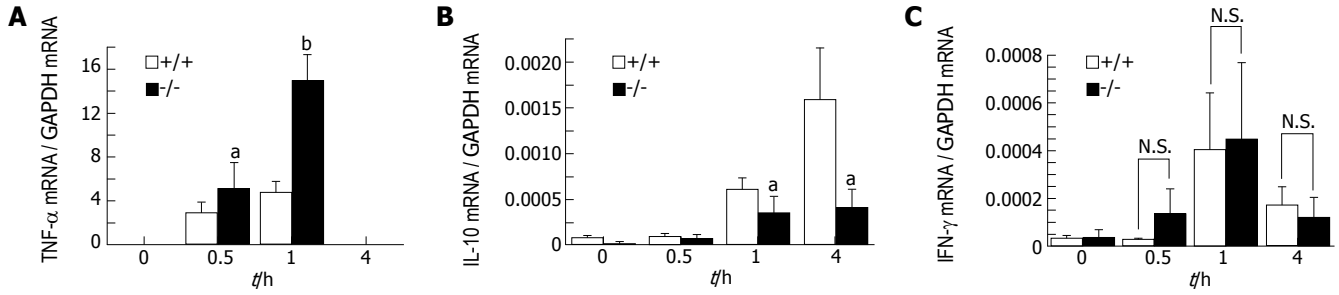


Figure 3 TNF-α, IL-10 and IFN-γ in the liver (**A-C**) of mice following GalN/LPS administration. (mean ± SE, $n = 6$). [a]$P < 0.05$, [b]$P < 0.01$, *vs* WT (Scheffé's test).

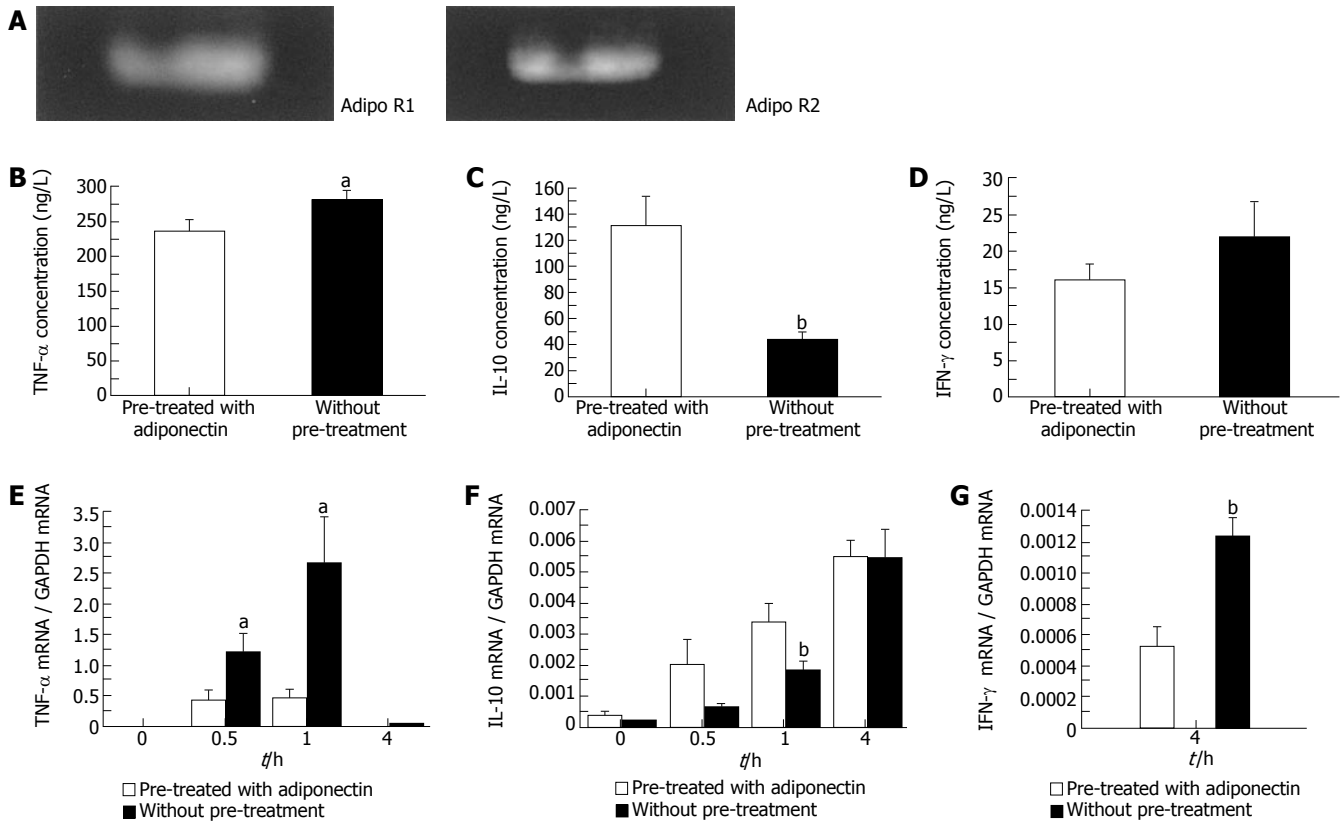


Figure 4 The expressions of adipoR1 and AdipoR2 in Kupffer cells (**A**). Effect of adiponectin on TNF-α, IL-10 and IFN-γ production by LPS-stimulated Kupffer cells *in vitro* (**B-D**). Pre-treated with or without adiponectin (10 mg/L) for 24 h followed by the stimulation with LPS (10 mg/L). TNF-α, IL-10 and IFN-γ mRNA expression in Kupffer cells after LPS stimulation (**E-G**). (mean ± SE, $n = 6$). [a]$P < 0.05$, [b]$P < 0.01$ (Scheffé's test).

$P < 0.05$; Figure 3B). The IFN-γ mRNA expression level in the liver increased, reaching peak levels 1 h after GalN/LPS administration, but no significant difference between adiponectin-/- mice and WT mice was found (Figure 3C). In the spleen, the IFN-γ mRNA expression levels also reached peak levels at 1 h after the administration, but there was no significant difference between adiponectin-/- mice and WT mice (data not shown).

Adiponectin receptors expression in Kupffer cell

We comfirmed the expressions of AdipoR1 and AdipoR2 in rat Kupffer cells using RT-PCR (Figure 4A).

Adiponectin inhibits LPS-induced TNF-α production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin led to a reduction in the levels of TNF-α released in response to LPS stimulation. TNF-α levels in the culture media of adiponectin-pretreated Kupffer cells were significantly lower than those for untreated cells 4 h after LPS stimulation ($P < 0.05$; Figure 4B). The LPS-induced TNF-α mRNA expression level in Kupffer cells was markedly suppressed by adiponectin pre-treatment. The expression level of TNF-α mRNA in Kupffer cells increased, reaching peak levels 1 h after LPS stimulation, but the levels at 0.5 and 1 h after the commencement of LPS stimulation in culture media of adiponectin-pretreated Kupffer cells was significantly lower than in those of untreated cells (at 0.5 h; $P < 0.05$, at 1 h; $P < 0.05$; Figure 4E).

Adiponectin increases LPS-induced IL-10 production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin increased the release of IL-10 in response to LPS stimulation.

IL-10 concentrations in the culture media of adiponectin-pretreated Kupffer cells were significantly higher at 4 h after LPS stimulation than those without adiponectin pre-treatment ($P < 0.01$; Figure 4C). The expression level of LPS-induced IL-10 mRNA in Kupffer cells was markedly increased as a result of adiponectin pre-treatment. The expression level of IL-10 mRNA in Kupffer cells was significantly higher in adiponectin-pretreated Kupffer cells 1 h after LPS stimulation than in untreated cells ($P < 0.05$; Figure 4F).

Lack of effect of adiponectin on LPS-induced IFN-γ production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin had no significant effect on IFN-γ concentrations in the culture media (Figure 4D). But the expression level of LPS-induced IFN-γ mRNA in Kupffer cells was significantly higher in adiponectin-pretreated Kupffer cells 4 h after LPS stimulation than in untreated cells. IFN-γ gene expression in Kupffer cells at 0 and 1h after LPS stimulation could not be determined quantitatively by RT-PCR ($P < 0.01$; Figure 4G).

DISCUSSION

The major findings of the present study were that a lack of adiponectin accelerates LPS-induced liver injury, and that adiponectin has an anti-inflammatory effect on Kupffer cells. Our results demonstrate that adiponectin-pretreated Kupffer cells produced less TNF-α and more IL-10 as a result of LPS treatment. These results suggest that adiponectin has an anti-inflammatory effect on LPS-treated Kupffer cells. An intraperitoneal injection of GalN/LPS in mice resulted in a more serious liver injury that was associated with a significantly higher mortality in adiponectin-/- mice than in WT mice. The plasma levels of TNF-α were significantly increased and those of IL-10 were significantly decreased in adiponectin-/- mice compared to WT mice, and TNF-α gene expression in the liver was significantly higher and those for IL-10 were lower in adiponectin-/- mice than in WT mice. These results indicate that a lack of adiponectin enhances LPS-induced liver injury and that the altered production of cytokines in Kupffer cells caused by a lack of adiponectin affect the severity of LPS-induced liver injury.

TNF-α causes liver cell apoptosis in GalN-sensitized mice[7,23]. Mice that are deficient in TNF-α receptors are protected against GalN/LPS-induced liver damage[24]. In our study, plasma TNF-α levels increased significantly in adiponectin-/- mice after GalN/LPS administration compared with WT mice, suggesting that a lack of adiponectin causes an over response of TNF-α production. Indeed, TNF-α gene expression in the liver increased significantly in adiponectin-/- mice after GalN/LPS administration compared with WT mice. Sennello *et al.* recently reported that adiponectin protects hepatocytes from TNF-α induced cell death[25]. Our results are in agreement with this conclusion. We previously reported that, in peripheral macrophages, adiponectin inhibits LPS-induced TNF-α production possibly through the suppression of TNF-α-induced IκB-α-NF-κB activation via the cAMP-dependent pathway[17,26]. In the present study, adiponectin pre-treated Kupffer cells showed a lower response to LPS than the controls. The TNF-α concentration in the culture media of adiponectin pre-treated Kupffer cells showed a significantly lower elevation than those of untreated cells, and TNF-α gene expression levels after the LPS stimulation of adiponectin pre-treated Kupffer cells were significantly lower than those of untreated cells. The TLR4 receptor system plays an important role in innate immunity[27]. Kupffer cells of mice express TLR4 mRNA and respond to LPS. The LPS-binding protein (LBP) complex associates with the CD14, and via TLR4, activates Kupffer cells to secrete TNF-α[7]. Adiponectin may inhibit this receptor system directly by suppressing IκB-α-NF-κB activation, although further studies will be needed to confirm this conclusion.

IL-10 exhibits a hepatoprotective role in GalN/LPS-induced liver injury by inhibiting the release of TNF-α[10,11,28]. In our study, plasma IL-10 levels were significantly diminished in adiponectin-/- mice after the administration of GalN/LPS compared with WT mice. GalN/LPS treatment of adiponectin-/- mice significantly reduced IL-10 gene expression in the liver compared with WT mice. Kupffer cells are known to release substantial amounts of IL-10 following LPS stimulation[9]. In our study, IL-10 concentrations in the culture medium of adiponectin pre-treated Kupffer cells showed a significantly higher response to LPS stimulation than the control cells. We recently reported that adiponectin increases IL-10 gene expression in human macrophages[18]. IL-10 gene expression in adiponectin pre-treated Kupffer cells was also significantly higher than in untreated cells. These data show that physiological concentrations of adiponectin are sufficient to induce IL-10 production in Kupffer cells in response to LPS stimulation, and that a lack of adiponectin resulted in a lower IL-10 response to LPS stimulation in Kupffer cells. These results suggest that adiponectin inhibits TNF-α production directly as well as indirectly through the induction of IL-10. Further studies will be required to elucidate the precise mechanism of this cross-talk.

IFN-γ is also involved in the toxic effects of LPS on the liver[29], and IFN-γ monoclonal antibodies reduce LPS-induced mortality in mice[30]. The overexpression of IFN-γ activates STAT-1 in the liver and promotes hepatocyte apoptosis following GalN/LPS administration[12]. IL-10 is reported to inhibit IFN-γ[31]. However, Sennelo *et al.* recently reported that, in a model of Concavaline A induced hepatitis, there was no significant difference in serum IFN-γ levels between lipodystrophic aP2-nSREBP-1c transgenic mice, with higher serum adiponectin, and *ob/ob* mice, with lower serum adiponectin levels[25]. Our study found no significant differences in plasma levels of IFN-γ between adiponectin-/- mice and WT mice, or IFN-γ gene expression levels in the liver and splenic lymphocytes between adiponectin-/- mice and WT mice. We found no significant difference in IFN-γ concentration in the culture media of adiponectin pre-treated Kupffer cells and untreated Kupffer cells. However, 4 h after the LPS stimulation, IFN-γ gene expression levels of adiponectin pre-treated Kupffer cells were significantly lower than

those of untreated cells. Further studies will be required to elucidate the effect of adiponectin on IFN-γ production in Kupffer cells.

Obesity is an independent risk factor for the development of chronic liver diseases such as NASH and alcoholic liver disease[1,2]. Serum adiponectin levels are decreased in obesity, and alcohol decreases the expression of adiponectin[13,14]. Adiponectin has been reported to confer protective effects against alcoholic liver injury, and hypoadiponectinemia is involved in the progression of non-alcoholic fatty liver disease (NAFLD) to steatohepatitis[32,33]. Our results suggest that the hypoadiponectinemia in obesity induces an altered response of Kupffer cells to LPS, and is possibly involved in the development of alcoholic liver disease and NASH. Adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) have been cloned by expression cloning[15]. AdipoR1 is expressed abundantly in skeletal muscle, and is also expressed in the liver. AdipoR2 is predominantly expressed in the liver. Thakur V *et al* showed the expressions of adipoR1 and R2 in Kupffer cells and we also found these expressions in Kupffer cells[34]. Recently Wolf AM *et al* reported the up-regulation of adiponectin in liver in ConA mediated acute liver failure[35]. In GalN/LPS induced acute liver injury, the expressions of adiponectin in the liver could not be determined quantitatively by RT-PCR (data not shown). To elucidate the effect of adiponectin produced in the liver in this model, further studies will be needed. Masaki *et al* reported that adiponectin prevents LPS-induced hepatic injury in a KK-Ay obese mice model[36]. KK-Ay obese mice show not only hypoadiponectinemia but also hyperglycemia and insulin resistance. Hyperglycemia and hyperinsulinemia should have an effect on the response of inflammatory cytokines to endotoxemia[37]. We used adiponectin-/- mice to rule out the effects of such other factors. In this model, we were able to clarify the direct effect of adiponectin deficiency on LPS-induced liver injury *in vivo*.

In conclusion, a deficiency of adiponectin could enhance LPS-induced liver injury possibly through modulation of cytokine production by Kupffer cells. The development of adiponectin receptor agonists or molecules that can induce adiponectin secretion might be helpful in controlling LPS-related liver diseases such as NASH and alcoholic liver injury.

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BASIC RESEARCH

Effect of solanine on the membrane potential of mitochondria in HepG2 cells and $[Ca^{2+}]_i$ in the cells

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Abstract

AIM: To observe the effect of solanine on the membrane potential of mitochondria in HepG2 cells and $[Ca^{2+}]_i$ in the cells, and to uncover the mechanism by which solanine induces apoptosis.

METHODS: HepG2 cells were double stained with AO/EB, and morphological changes of the cells were observed using laser confocal scanning microscopy (LCSM). HepG2 cells were stained with TMRE, and change in the membrane potential of mitochondria in the cells were observed using LCSM. HepG2 cells were double stained with Fluo-3/AM, and change of $[Ca^{2+}]_i$ in the cells were observed using LCSM. HepG2 cells were double stained with TMRE and Fluo-3/AM, and both the change in membrane potential of mitochondria and that of $[Ca^{2+}]_i$ in the cells were observed using LCSM.

RESULTS: Cells in treated groups showed typical signs of apoptosis. Staining with TMRE showed that solanine could lower membrane potential; staining with Fluo-3/AM showed that solanine could increase the concentration of Ca^{2+} in tumor cells; and those of double staining with TMRE and Fluo-3/AM showed that solanine could increase the concentration of Ca^{2+} in the cells at the same time as it lowered the membrane potential of mitochondria.

CONCLUSION: Solanine opens up the PT channels in the membrane by lowering the membrane potential, leading to Ca^{2+} being transported down its concentration gradient, which in turn leads to the rise of the concentration of Ca^{2+} in the cell, turning on the mechanism for apoptosis.

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Key words: Solanine; Hepatocarcinomatic cell; Ca^{2+} in the cell; Membrane potential; Laser confocal scanning microscopy

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INTRODUCTION

Solanine is found mainly in the tuber of the potato (Solanum tuberosum L.)[1-5] and in the whole plant of the nightshade (Solanum nigrum Linn) of the family Solanaceae. The content of this substance is rather high in the green peel and the sprouts of potato and is the main toxic substance[6]. The whole plant of the nightshade contains many steroid alkaloids, including solamargine, solasonine, and solanine, as well as saponin and other substances. It can be used for anti-tumor purposes, with a strong inhibitory effect on tumors in animals and a clearly toxic effect on tumor cells[7]. Its ethanol extract is capable of inhibiting the growth of breast cancer and induce apoptosis in tumor cells[8]. The extract from the nightshade also has a strong anti-inflammatory effect because it can facilitate the formation of antibodies[9]. The anti-tumor effect of solamargine has been reported[10], but there is as yet no report about any anti-tumor effect of solanine. From our past experience in both in vivo and in vitro experiments, we have found that solanine is cytotoxic to cells, especially for the hepatocarcinomatic cell HepG2. Through morphological observation, as well as DNA ladder and flow cytometry, we discovered that solanine exerted its anti-tumor effect by inducing apoptosis in HepG2.

The concept "apoptosis" was officially proposed by Kerr in 1972[11]. In the three decades since it was proposed, it has always been the focus of biological researches. Especially since 1990s, the study of apoptosis has suddenly been propelled to the frontier of life science, leading to an upsurge of research activities involving almost all the fields of biomedical studies. As a result, a great deal of literature accumulated in a few years about the

morphological characteristics of apoptosis, biochemical changes in apoptotic cells, genes regulating apoptosis, and various factors that can lead to apoptosis, as well as the relationship between apoptosis and health conditions. Membrane potential of mitochondria[12,13] and $[Ca^{2+}]_i$ in the cells[14,15], two factors closely associated with apoptosis, are themselves supplementary to and closely related with each other.

Rapid and continuous increase in the concentration of calcium ion is the earliest detectable biochemical change in cells undergoing apoptosis. The fact that the use of calcium-free culture medium and other methods capable of inhibiting the rise in the concentration of Ca^{2+} in cells can help to inhibit apoptosis[16] also shows that the rise in the concentration of Ca^{2+} in cells is an important condition for apoptosis to take place. A typical sign of apoptosis is the appearance of apoptotic bodies, but it is only when the concentration of Ca^{2+} has been raised that endogenous nuclease is activated, cutting the DNA chain between nucleosomes into discontinuous fragments (or apoptotic bodies), the basic unit of which is 180-200 bp long.

The mitochondrion is the storehouse for calcium in the cell, and change in its membrane potential can lead to Ca^{2+} and other ions being released from the mitochondrion or flowing into it, and the release and influx of Ca^{2+} and other ions are also the direct causes for changes in the membrane potential of mitochondrion. The essence of membrane potential change is the opening of the PT channels in the membrane. The opening of these channels precedes the release of Ca^{2+}, which is followed by changes in the membrane potential.

There are many approaches to studying apoptosis. In the present study, we tried to explicate the mechanism by which solanine induced apoptosis of tumor cells by focusing on the mitochondria and by observing the effect of solanine on the membrane potential of $HepG_2$ and $[Ca^{2+}]_i$ in the cell(Figure 1).

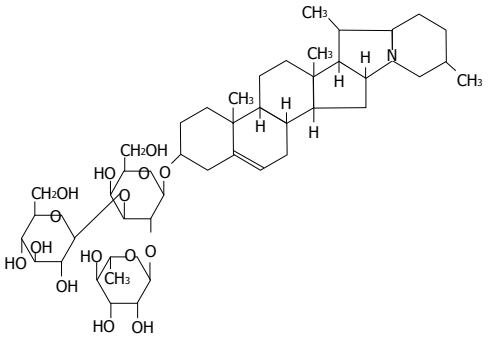


Figure 1 Molecular structure of solanine.

MATERIALS AND METHODS

Materials

The cell line: $HepG_2$ human hepatocarcinomatic cell lines were purchased from the Institute for Cancer Research, Heilongjiang Cancer Hospital.

Test drugs: Solanine with a purity of 99% was provided by Heilongjiang Institute For Drug Control.

Chemical reagents: RPMI 1640 culture medium was supplied from HyClone Laboratories; fetal bovine serum from Hangzhou Sijiqing Biological Engineering Materials Co.,Ltd; pancreatin from Gibio; ethidium bromide (EB) from AMRESCO; acridine orange (AO) from AMRESCO; Fluo-3/AM from Biotium, US; TMRE(molecular probe) from Biotium, US; HEPES from AMRESCO.

Preparation of the reagents: Fluo-3/AM 0.1 mg/stick was diluted to suitable concentration with DMSO, and then separately loaded and stored in the dark. TMRE 25 mg/stick was made into 1 mg/mL mother solution using anhydrous alcohol, separately loaded and stored in the dark. These were diluted with buffer solution for laboratory use and added to achieve the needed final concentrations before they were used in experiments.

Apparatuses: CO_2 incubator (CO-150, NBS, US); inverted microscope (CKX-41-32, Olympus, Japan); superclean bench (SW-CJ-2F, Suzhou Purification Equipments of the Sujing Group, Suzhou); laser confocal scanning microscope (SP2, Leica, Germany) were used.

Methods

Cell culture and treatment: $HepG_2$ cell culture was incubated in RPMI 1640 medium containing 10% fetal bovine serum at 50 mL/L CO_2 and 37℃, and transfer of culture was performed once every 3-4 d. When the cells grew steadily in the phase of logarithmic growth, 0.25% pancreatin was used to digest the cells. Digested cells were re-suspended using RPMI 1640 medium containing 10% fetal bovine serum and counted, and the concentration of cells was adjusted to 1×10^4 /mL. Cell suspension was added to the round troughs of 35 mm Petri dishes at 200 μL/dish, and the dishes were divided into 2, 0.4, 0.08, 0.016, and 0.0032 μg/mL treatment groups, the control, and the positive control (to be treated with camptothecine). After being incubated for 24 h at 50 mL/L CO_2 and 37℃, the treatment groups were treated with solanine of different concentrations so that their final concentrations were 2, 0.4, 0.08, 0.016, and 0.0032 μg/mL, respectively; an equal volume of RPMI 1640 was added to the control, while the positive control was treated with camptothecine, with a final concentration of 0.08 μg/mL. The dishes were then incubated for another 48 h at 50 mL/L CO_2 and 37℃ in CO_2 incubator.

Observation of Solanine-induced morphological changes of $HepG_2$ nuclei using LCSM: After 48 h, the cells were taken out of the incubator. The culture solution in the Petri dishes was sucked out, and the cells were rinsed 3 times with PBS at 5 min/time, double stained with 200 μL of acridine orange (AO)/ethidium brumide (EB) so that the final concentration was 5 μg/mL, incubated for 5-10 min at 37℃, and rinsed 3 times with PBS, 5 min/time. Then 200 μL of PBS was added, and LCSM was used to observe the morphology of the cells. Dual-channel activation was used, with an excitation wavelength of 488 nm and emission wavelength of 500-520 nm for PMT1(AO) and an excitation wavelength of 543 nm and emission wavelength of 600-700 nm for PMT2(EB)[17]. Other devices included Objective APO CS40 × /1.25 oil, zoom > 1, pinhole 1.5 Airy, mode XYZ, format 512 × 512.

Observation of Solanine-induced change in $[Ca^{2+}]_i$

in $HepG_2$ cells using LCSM: After 48 h, the cells were taken out of the incubator. The culture solution in the Petri dishes was sucked out, and the cells were rinsed 3 times with HEPES, and 4 μg/mL Fluo-3/AM (Molecular Probes) was added, 200 μL/dish. After the cells were incubated at 37℃ for 50 min and rinsed with HEPES for 3 times, 200 μL of HEPES culture medium was used to cover all the cells in the troughs, and then LCSM was used to observe the fluorescence intensity (FI) of the cells, with an excitation wavelength of 488 nm and emission wavelength of 555 ± 15 nm. Other devices included objective APO CS40 ×/1.25 oil, zoom > 1, pinhole 1.5 Airy, mode XYZ, format 512 × 512.

Observation of Solanine-induced change in the membrane potential of the mitochondria in the cells using LCSM: After 48 h, the cells were taken out of the incubator. The culture solution in the Petri dishes was sucked out, the cells were rinsed 2-3 times with PBS, and 200 μL of tetramethyl rhodamine ethyl ester (TMRE, Molecular Probes) was gently added to the dishes, so that the final concentration was 2 μmol/L (1.03 μg/mL). The cells were then incubated for 30 min and, after the staining solution was sucked out, gently rinsed 3 times with PBS. 200 μL PBS was used to cover all the cells in the troughs, and then LCSM was used to observe the fluorescence intensity of the cells, with an excitation wavelength of 543 nm and emission wavelength of 570 ± 20 nm. Other devices included objective APO CS40 ×/1.25 oil, zoom > 1, pinhole 1.5 Airy, mode XYZ, format 512 × 512.

Simultaneous observation of changes in the morphology of cells, membrane potential, and the concentration of calcium in the cells: Experiment was performed on 2 groups of $HepG_2$ cells simultaneously. Cell incubation, treatment, and operation procedures were all the same as above, with the same amount of time and conditions for cell incubation. One group was marked with AO/EB and the effect of solanine on its $HepG_2$ cells was observed using LCSM; while $HepG_2$ cells in the other group were marked with TMRE and Fluo-3/AM, and changes in membrane potential of the mitochondria and $[Ca^{2+}]_i$ in the cells were observed using LCSM. Methods for AO/EB double staining and measurement were as before, while the procedures for double staining with Fluo-3/AM and TMRE and measurement are as follows: (1) Staining after 48 h, the cells were taken out of the incubator. The culture solution in the Petri dishes was sucked out, the cells were rinsed 3 times with RPMI1640, and 150 μL of tetramethyl rhodamine ethyl ester (TMRE, Molecular Probes) working fluid with a concentration of 2 μmol/L (1.03 μg/mL) was pipetted onto the dishes. The cells were then incubated for 30 min at 37℃, and after the staining solution was sucked out, rinsed 3 times with RPMI1640. Finally 150 μL of Fluo-3/AM (Molecular Probes) with a concentration of 4 μg/mL was added. The cells were then incubated for 50 min at 37℃ and rinsed 3 times with RPMI1640, and 200 μL of RPMI1640 was added to cover all the cells in the troughs, whereupon the Petri dishes were mounted for measurement. (2) Observation LCSM was used to examine the fluorescence intensity (FI) of the cells. Dual-wavelength excitation was used, with excitation wavelengths of 488 nm and 543 nm, respectively. The emission wavelength used for PMT1 was 555 ± 15 nm, while that for PMT2 was 570 ± 20 nm. Other devices included Objective APO CS40 × /1.25 oil, zoom > 1, pinhole 1.5 Airy, mode XYZ, format 512 × 512. Sequential scanning was used to eliminate interference due to spectral overlap.

Statistical analysis

t test was performed on data obtained from the experiments, with the results expressed in the format mean ± SD. $P < 0.05$ was taken as significant.

RESULTS

Effect of Solanine on the morphology of $HepG_2$ cells

After the DNA-specific fluorochromes AO and EB were used to stain $HepG_2$ cells, LCSM was used to observe the morphological changes of $HepG_2$ cells treated with solanine. From Figure 2A, it can be seen that the control was morphologically normal. The nuclei of different cells were of similar sizes, regularly shaped, and evenly stained. The more deeply colored parts of the nuclei represented heterochromatin which did not take part in transcription under normal circumstances. With solanine treatment, however, the cells showed marked morphological changes. In the groups treated with 0.0032 μg/mL and 0.016 μg/mL of solanine (Figures 2B and 2C, respectively), the cells were wrinkled, and the chromatin was concentrated and marginalized. In the group treated with 0.08 μg/mL of solanine (Figure 2D), cells with fragments and apoptotic bodies appeared, a typical sign for apoptosis. In the groups treated with 0.4 μg/mL and 2 μg/mL of solanine (Figures 2E and 2F, respectively), the number of cells containing apoptotic bodies was significantly increased. At the same time, it could be seen that high dosage of solanine was lethal to tumor cells, because with increasing dosage, the number of viable cells decreased significantly. Apoptotic bodies also appeared clearly in the positive control treated with camptothecin with the final concentration of 0.08 μg/mL.

Change induced by Solanine in $[Ca^{2+}]_i$ in $HepG_2$ cells in the process of apoptosis

As shown in Figure 3 the effect of different dosages of solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL) on $[Ca^{2+}]_i$ in $HepG_2$ cells was observed using LCSM with staining with the fluorescent probe Fluo-3/AM. On the left side of each group is the photograph (green) showing $[Ca^{2+}]_i$ taken under the confocal microscope, with the depth of the color representing the fluorescence intensity (FI). On the right is the 3D configuration reconstructed using LCSM for the group, with different colors reflecting different FI values, which indirectly reflect the concentrations of $[Ca^{2+}]_i$ for the test group in question. It is shown from these photographs that the $[Ca^{2+}]_i$ for the control in Figure 3A was very low, while those for the groups treated with solanine (Figures 3B-F) were all increased, in a concentration dependent manner. The positive control camptothecin did not increase $[Ca^{2+}]_i$. Statistics on FI obtained through LCSM for the various groups in Table 1 showed the same results.

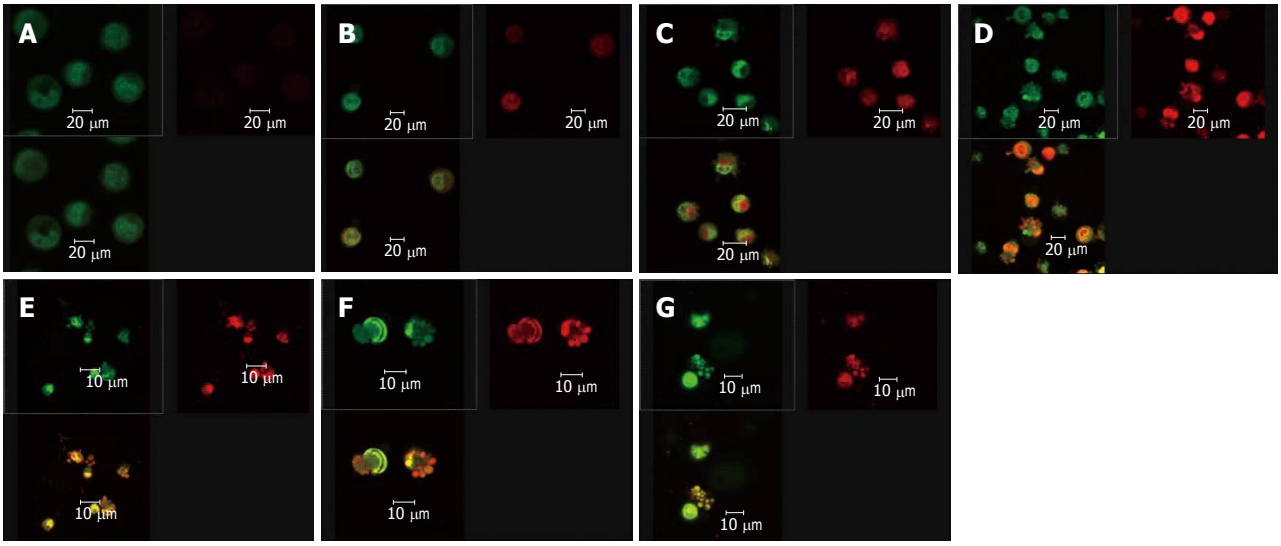


Figure 2 Effect of solanine on the morphology of HepG2. **A**: Control group, PMT1 nuclear chromatin was stained green and showed normal structure, while PMT2 showed no or only weak red fluorescence; **B**: Group treated with Solanine with final concentration of 0.0032 μg/mL: The cells show slightly pyknotic and crumb-shaped structures. Permeability of cell membrane has increased, so that AO and EB can enter the cells, resulting in the cells showing both green and red fluorescence; **C**: Group treated with Solanine with final concentration of 0.016 μg/mL: The cells show slightly pyknotic and crumb-shaped structure. Fragments or apoptotic bodies appear in the nuclei of some of the cells; **D**: Group treated with solanine with final concentration of 0.08 μg/mL: Cell structure is further damaged. The cells are not only stained with AO and EB, but morphologically the nuclei have become deeply stained fragments or apoptotic bodies; **E**: Group treated with solanine with final concentration of 0.4 μg/mL: Cell structure is damaged even further. Apoptotic bodies have definitely appeared; **F**: Group treated with solanine with final concentration of 2 μg/mL: Definite apoptotic bodies can be seen, while the number of cells in sight has decreased; **G**: Group treated with camptothecin with final concentration of 2 μg/mL: The appearance of apoptotic bodies is obvious in HepG2 cells.

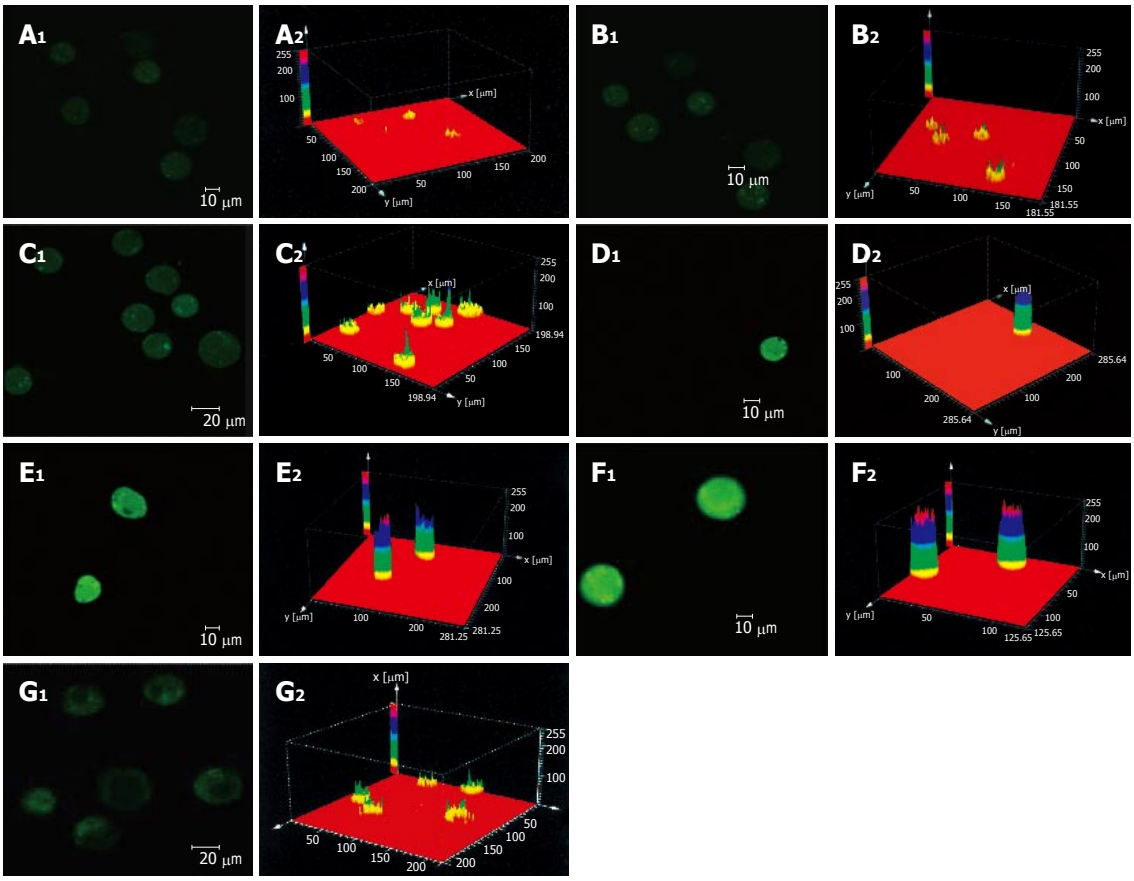


Figure 3 Effect of Solanine on $[Ca^{2+}]_i$ in HepG2 cells. **A**: Control; **B**: 0.0032 μg/mL solanine; **C**: 0.016 μg/mL solanine; **D**: 0.08 μg/mL solanine; **E**: 0.4 μg/mL solanine; **F**: 2 μg/mL solanine; **G**: 0.08 μg/mL camptothecin.

Change induced by Solanine in the membrane potential of mitochondria in HepG2 cells in the process of apoptosis

The effect of different dosages of solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL) on the membrane potential of mitochondria in HepG2 cells was observed using LCSM with staining by the fluorescent probe TMRE (Figure 4). The brightness of the coloration reflected different values of FI, thus indirectly indicating the membrane potential of mitochondria in the cells for these different groups. From those photographs, it could be seen that the membrane

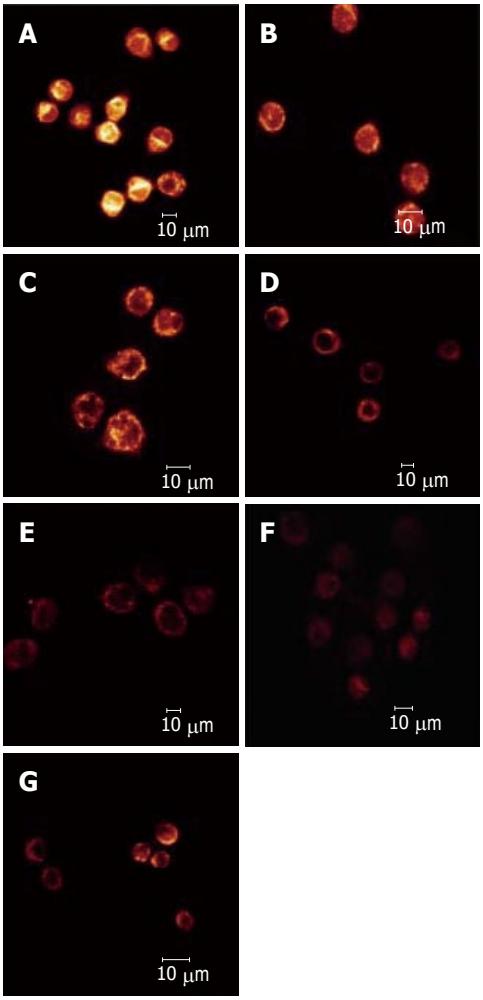


Figure 4 Effect of Solanine on membrane potential of mitochondria in $HepG_2$ cells. **A**: Control; **B**: 0.0032 μg/mL solanine; **C**: 0.016 μg/mL solanine; **D**: 0.08 μg/mL solanine; **E**: 0.4 μg/mL solanine; **F**: 2 μg/mL solanine; **G**: 0.08 μg/mL camptothecin.

Table 1 Effect of Solanine on $[Ca^{2+}]_i$ in $HepG_2$ cells (mean ± SD)

Group	Final concentration (μg/mL)	Number of cells (*n*)	FI
Control	-	18	23.98 ± 10.06
Camptothecin group	0.08	16	26.92 ± 8.20
Solanine groups	0.003	25	40.31 ± 10.15[b]
	0.016	37	59.31 ± 17.56[b]
	0.08	17	91.01 ± 23.19[b]
	0.4	19	158.75 ± 14.52[b]
	2	20	209.85 ± 10.86[b]

Comparison with blank: [b]$P < 0.01$.

Table 2 Effect of solanine on the membrane potential of mitochondria in $HepG_2$ cells (mean ± SD)

Group	Final concentration (μg/mL)	Cell number (*n*)	FI
Control	-	53	75.70 ± 31.24
Camptothecin group	0.08	16	47.17 ± 15.98
Solanine groups	0.003	66	70.04 ± 17.83
	0.016	90	63.21 ± 16.36[b]
	0.08	144	52.26 ± 26.46[b]
	0.4	37	32.50 ± 19.99[b]
	2	33	28.49 ± 9.71[b]

Comparison with blank: [b]$P < 0.01$.

potential of mitochondria in the cells was relatively high for the control in Figure 4A, while those for the groups treated with solanine (Figures 4B-F) were all increased, in a dosage dependent manner. Statistics on FI obtained through LCSM for the various groups in Table 2 showed that the membrane potential of mitochondria in the cells was significantly lowered ($P < 0.01$) in groups treated with 0.016, 0.08, 0.4, and 2 μg/mL of solanine. The membrane potential in the 0.0032 μg/mL group was also lowered, but the difference from the control was not significant. The membrane potential of mitochondria in $HepG_2$ cells of the group treated with 0.08 μg/mL of camptothecin was also significantly lowered.

Simultaneous observation of changes induced by solanine in cell morphology, $[Ca^{2+}]_i$ in cells, and mitochondrial membrane potential in $HepG_2$ cells in the process of apoptosis

Double staining with AO/BE and with Fluo-3/AM and TMRE were simultaneously used in this experiment (Figure 5). Changes in cell morphology, $[Ca^{2+}]_i$ in cells, and membrane potential of mitochondria in the same cell were simultaneously observed using LCSM for groups treated with different concentrations of solanine, so as to examine simultaneous changes in cell morphology, $[Ca^{2+}]_i$ in cells, and membrane potential of mitochondria in cells at the same time points in the apoptosis process induced by solanine of different concentrations and, moreover, to explore the physiological mechanisms of $[Ca^{2+}]_i$ and membrane potential of mitochondria in the cells and the relationship between them. As shown in Figure 5A for the control group, the cells were normal morphologically, and the $[Ca^{2+}]_i$ in the cells was relatively low, while the corresponding mitochondrial membrane potential was relatively high. Results for the groups treated with solanine (with final concentration of 0.0032, 0.016, 0.08, 0.4, and 2 μg/mL, respectively) are shown in Figures 5B-F. It could be seen that with the dosage increased, the shape of the cells gradually became more and more irregular, and typical apoptotic cells such as irregularly shaped cells and apoptotic bodies appeared, with apoptotic bodies, characteristic of apoptosis appearing first in the group treated with 0.08 μg/mL of solanine (Figure 5D). As the dosage increased, the percentage of apoptotic bodies gradually increased, and $[Ca^{2+}]_i$ in the cells also gradually increased, while the membrane potential of mitochondria in the cells gradually decreased for the corresponding groups. The group treated with camptothecin showed no change in $[Ca^{2+}]_i$ but it did show a significantly decreased membrane potential of mitochondria, results consistent with those obtained with single staining. It also showed that solanine induced apoptosis in $HepG_2$ cells by a

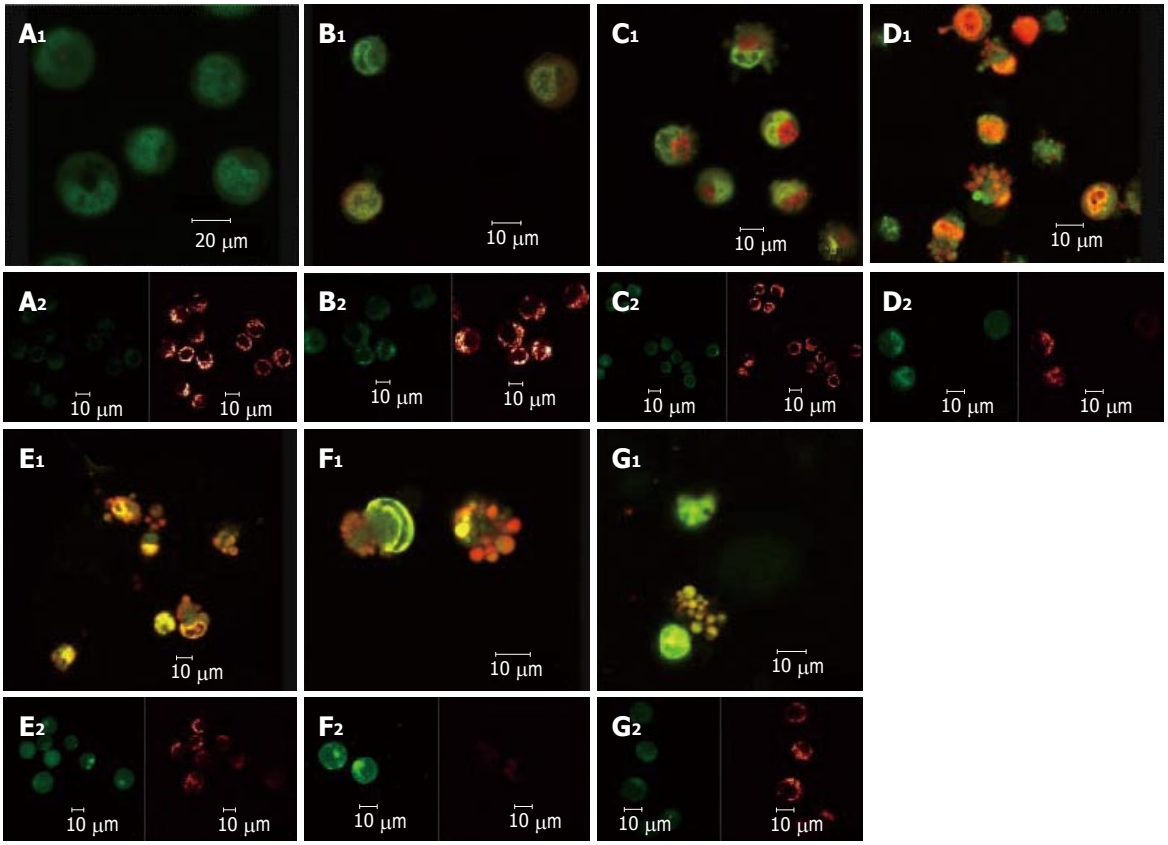


Figure 5 Changes induced by solanine in cell morphology, $[Ca^{2+}]_i$ in cells, and membrane potential of mitochondria in $HepG_2$ cells in the process of apoptosis. **A**: Control; **B**: 0.0032 μg/mL solanine; **C**: 0.016 μg/mL solanine; **D**: 0.08 μg/mL solanine; **E**: 0.4 μg/mL solanine; **F**: 2 μg/mL solanine; **G**: 0.08 μg/mL camptothecin.

Table 3 Effect of solanine on $[Ca^{2+}]_i$ in $HepG_2$ cells and mitochondrial membrane potential (mean ± SD)

Group	Final concentration (μg/mL)	Number of cells	Fluo-3/AM-FI	TMRE-FI
Control	-	111	24.96 ± 4.03	51.85 ± 19.18
Camptothecin group	0.08	21	28.80 ± 7.01	25.50 ± 5.76[b]
Solanine groups	0.003	49	33.85 ± 9.30[a]	39.52 ± 15.41[b]
	0.016	33	34.37 ± 14.18[a]	35.01 ± 11.12[c]
	0.08	36	42.73 ± 14.75[b]	28.13 ± 4.31[d]
	0.4	39	52.80 ± 11.07[b]	23.21 ± 12.22[d]
	2	37	58.62 ± 16.04[b]	13.09 ± 4.71[d]

Comparison with blank: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.005$; [d]$P < 0.001$.

different mechanism from camptothecin. Detailed changes of different groups are shown in Table 3.

Solanine-induced changes in $[Ca^{2+}]_i$ and membrane potential of mitochondria in $HepG_2$ cells in the process of apoptosis and their distribution in the cells

When the images for the double staining with Fluo-3/AM and TMRE were recombined using the image analyzing capacity of the laser confocal scanning microscope, it could be seen that Ca^{2+} was widely distributed in the whole cell, but with different concentrations in different regions of the cell. In contrast, membrane potential of mitochondria was distributed only in certain parts of the cell (Figure 6). Superimposing the two images, it can be seen that regions where Ca^{2+} was more concentrated were basically also those where membrane potential was distributed.

DISCUSSION

The mitochondrion is an important organelle of the eukaryotic cell and the main site for the production of ATPs in animal cells[18,19]. NADH is produced from the tricarboxylic cyclophorase series in mitochondrial matrix through the dehydroxidization of the substrate, and then oxidized through the electron transport chain on the inner membrane of the mitochondrion. This leads to the transmembrane transfer of protons, resulting in a transmembrane proton gradient (or transmembrane potential). The ATP synthetase on the inner membrane of the mitochondrion synthesizes ATPs using the energy derived from the proton gradient. Through the ADP/ATP carriers on the inner membrane of mitochondria, ATPs synthesized this way are exchanged for ADPs from the cytoplasm, thus entering the cytoplasm to take part in various processes requiring energy.

In recent years, reports have been made that the dissipation of the membrane potential of mitochondria precedes the activation of nuclease[20], and that it precedes the exposure of phosphatidylserine on the surface of the cell. When the transmembrane potential of mitochondria is dissipated, the cell would enter the irreversible apoptotic process. The uncoupled respiratory chain of the mitochondrion would produce a large amount of active oxygen, oxidizing the cardiolipin on the inner membrane of the mitochondrion. Experiments have shown that the decoupling reagent mClCCP can lead to the apoptosis of

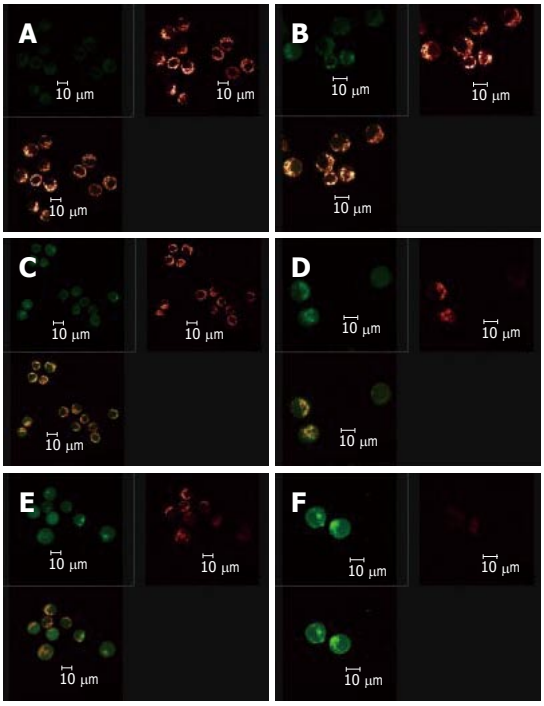


Figure 6 Distribution of solanine-induced changes in $[Ca^{2+}]_i$ and membrane potential of mitochondria in the $HepG_2$ cells in the process of apoptosis. **A**: Blank; **B**: 0.0032 μg/mL solanine; **C**: 0.016 μg/mL solanine; **D**: 0.08 μg/mL solanine; **E**: 0.4 μg/mL solanine; **F**: 2 μg/mL solanine.

lymphocytes[21,22], whereas if the transmembrane potential of mitochondria can be stabilized, apoptosis would be prevented. Therefore, the dissipation of transmembrane potential of mitochondria is closely related to apoptosis.

The dissipation of transmembrane potential of mitochondria in the process of apoptosis is mainly caused by changes in the permeability of the inner membrane of mitochondria, which is due to the formation of dynamic pores for permeability transition (PT) (each composed of several proteins) at points of contact between the outer and inner membranes of the mitochondrion[23]. PT channels are made up of proteins from various parts of the mitochondrion and those from the cytoplasm[24,25]. They include a protein in the cytochylema (hexokinase)[25], proteins on the outer membrane of the mitochondrion (peripheral benzodiazepine receptors and voltage-dependent anion channels), a protein in the interstice between the inner and outer membranes of the mitochondrion (creatine kinase)[26], a protein on the inner membrane of the mitochondrion (the ADP-ATP carrier)[27], and a protein in the mitochondrial matrix (cyclophilin D)[28]. Any substance, such as protoporphyria IX, the ligand for benzodiazepine receptors, that can specifically induce the formation of PT channels in mitochondria could cause apoptosis[29-32]. PT channels are a kind of channels with high electric conductivity. The lowering or even disappearance of membrane potential would suggest that PT channels are open.

Results from earlier experiments show that solanine can induce the apoptosis of $HepG_2$ cells. In the present study, double staining with AO/EB and solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL) carried by TMRE were used to treat $HepG_2$ cells, and LCSM was used to observe changes in the membrane potential of mitochondria as solanine induced the appearance of apoptotic bodies[33]. As solanine induced the appearance of apoptotic bodies and caused the apoptosis of cells, the membrane potentials of mitochondria in cells in groups treated with different dosages of solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL) were all decreased in comparison with the control (Figures 2 and 4, and Table 2). The amount of decrease was dosage-dependent, and the difference from that of the control was significant for the groups treated with 0.016, 0.08, 0.4, and 2 μg/mL of solanine, respectively, suggesting that solanine can lead to the lowering of the membrane potential of mitochondria in $HepG_2$ cells. However, the lowering of the membrane potential of mitochondria is a manifestation of the opening of the PT pores of the mitochondria[34], so solanine can lead to the opening of the PT pores of the mitochondria.

The opening of PT pores can lead to the occurrence of two events[35]: (1) the intra-membrane and extra-membrane ion concentrations tend toward equilibrium, the transmembrane H^+ gradient disappears, and the respiratory chain is uncoupled; (2) the flow of intra- and extra-membrane ions toward equilibrium leads to a hypertonic mitochondrial matrix, resulting in change in the volume of the mitochondrion. Since the surface area of the folded inner membrane of mitochondrion is larger than that of the outer membrane, this would lead to the rupture of the outer membrane.

These two events lead respectively to two consequences. Since a large number of Ca^{2+} have accumulated in mitochondria, the concentration of Ca^{2+} in mitochondria is much higher than that in the cytoplasm. The occurrence of the first event would lead directly to the flow of Ca^{2+} from the mitochondria to the cytoplasm, resulting in rapid increase in the concentration of Ca^{2+} in the cytoplasm. In the present study, the Ca^{2+}-specific molecular probe Fluo-3/AM was used to carry solanine at different concentrations to treat $HepG_2$ cells, and LCSM was used to observe changes in $[Ca^{2+}]_i$ in the cells after the treatment. In groups treated with different dosages of solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL), the concentrations of Ca^{2+} in $HepG_2$ cells were all increased in a dosage-dependent way (Figure 3 and Table 1). This verifies the inference we have drawn, namely, by opening up PT channels, solanine leads to the release of Ca^{2+} from the mitochondria, resulting in the increase of $[Ca^{2+}]_i$ in the cell. In order to obtain more powerful evidence for our inference, LCSM with double staining with Fluo-3/AM and TMRE was used to observe simultaneously the change in membrane potential and the change in $[Ca^{2+}]_i$ in the cell after the treatment. The results showed that as the dosage increased, the rate of apoptosis gradually increased, the membrane potential gradually decreased, and $[Ca^{2+}]_i$ in the cell gradually increased (Figure 5 and Table 3). These results were consistent with that obtained with single staining.

At the same time, the data collected using LCSM was analyzed and observation was made on the distribution of $[Ca^{2+}]_i$ and the membrane potential of mitochondria in the cells in the apoptotic process of $HepG_2$ induced by solanine. The image overlay function of LCSM was used

to superimpose and compare the images of $[Ca^{2+}]_i$ and the membrane potential of mitochondria in the cells. Results showed that membrane potential was mainly distributed on the inner membrane of the cells, with no distribution in the center of the cells; but the inner membrane was precisely where mitochondria were concentrated (Figure 6). $[Ca^{2+}]_i$ was distributed throughout the whole cell, but the distribution was uneven. This can be observed in the photograph with green fluorescence in Figure 6, where the brightness of the fluorescence depends on the region of the cell. When the image for $[Ca^{2+}]_i$ distribution (green) was superimposed on that for membrane potential (red), it can be seen that regions where Ca^{2+} was concentrated overlaps with where membrane potential was concentrated (as shown by the yellow regions in the third photograph in each group in Figure 6), suggesting that the distribution of Ca^{2+} in the mitochondria was high. In addition, the concentration of Ca^{2+} in both the mitochondria and the cytoplasm continued to rise in the different groups, suggesting that as $[Ca^{2+}]_i$ increased in the cytoplasm, Ca^{2+} flew out not only from the mitochondria, but also from some other source(s) as well, e.g., an inflow of Ca^{2+} from outside the cell.

In conclusion, solanine can facilitate the opening of the PT channels in mitochondria, leading to release of Ca^{2+} from these organelles. This results in an increase in the concentration of Ca^{2+} in the cell, thus triggering the mechanism for apoptosis and the occurrence of apoptosis. However, to uncover how the PT channels are opened and which enzymes and genes are involved in this process, further research is needed.

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BASIC RESEARCH

Dynamic functional and ultrastructural changes of gastric parietal cells induced by water immersion-restraint stress in rats

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Abstract

AIM: To investigate the dynamic functional and ultrastructural changes of gastric parietal cells induced by water immersion-restraint stress (WRS) in rats.

METHODS: WRS model of Sprague-Dawley (SD) rats was established. Fifty-six male SD rats were randomly divided into control group, stress group and post-stress group. The stress group was divided into 1, 2 and 4 h stress subgroups. The post-stress group was divided into 24, 48 and 72 h subgroups. The pH value of gastric juice, ulcer index (UI) of gastric mucosa and H^+, K^+-ATPase activity of gastric parietal cells were measured. Ultrastructural change of parietal cells was observed under transmission electron microscope (TEM).

RESULTS: The pH value of gastric juice decreased time-dependently in stress group and increased in post-stress group. The H^+, K^+-ATPase activity of gastric parietal cells and the UI of gastric mucosa increased time-dependently in stress group and decreased in post-stress group. Compared to control group, the pH value decreased remarkably (P = 0.0001), the UI and H^+, K^+-ATPase activity increased significantly (P = 0.0001, P = 0.0174) in 4 h stress subgroup. UI was positively related with stress time (r = 0.9876, P < 0.01) but negatively with pH value (r = -0.8724, P < 0.05). The parietal cells became active in stress group, especially in 4 h stress subgroup, in which plenty of intracellular canalicular and mitochondria were observed under TEM. In post-stress group, the parietal cells recovered to resting state.

CONCOUSION: The acid secretion of parietal cells is consistent with their ultrastructural changes during the development and healing of stress ulcer induced by WRS and the degree of gastric mucosal lesions, suggesting gastric acid play an important role in the development of stress ulcer and is closely related with the recovery of gastric mucosal lesions induced by WRS.

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Key words: Stress ulcer; Gastric parietal cells; Ultrastructure; H^+, K^+-ATPase activity; Sprague-Dawley rat

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INTRODUCTION

Stress ulcer is a common complication of severe brain trauma and psychosis in clinical practice. Critically ill patients are at increased risk of developing stress-related gastric mucosal lesions and gastrointestinal bleeding[1,2]. But the pathogenesis of stress-related gastric mucosal lesions is not fully elucidated. The development and healing of stress ulceration are a complex process affected by many factors. It had been shown that gastric acid plays an important role in the development of stress-induced ulcer and is the most common endogenous destructive factor[3-5].

It is reported that water immersion-restraint stress (WRS) may lead to changes of gastric acid secretion. Some studies indicate that WRS increases acid secretion[6,7], while other studies showed that gastric acid secretion decrease during WRS[8,9]. Gastric acid secretion is intimately related with the ultrastructure of parietal cells, but the dynamic ultrastructure change of parietal cells during WRS has not been reported yet.

Moreover, changes of parietal cells ultrastructure and gastric acid secretion during the healing of stress-induced ulceration are poorly understood so far. The purpose of the present study was to investigate the dynamic ultrastructural and functional of gastric parietal

cells induced by WRS in order to demonstrate the role of gastric acid in the development of stress ulcer and in the healing of gastric mucosal lesions.

MATERIALS AND METHODS

Animals and water immersion-restraint stress model

Fifty-six male SD rats, weighing 180-220 g were purchased from Xipuer-Bikai Experimental Animal Co. LTD, Shanghai, China. The animals were fasted for 24 h with free access to water and deprived of water 1 h before the experiment. Animals were randomly divided into control group (n = 8), stress group (n = 24) and post-stress group (n = 24), and exposed to various periods of stress and immersed in 19 ± 1℃ water as previously described[10]. Rat in control group were sacrified after lightly anesthetized with ether. Rats in stress group were killed after 1, 2 and 4 h of WRS respectively. Rats in post-stress group were killed after 24, 48 and 72 h of WRS respectively[11].

Measurement of gastric juice pH

All rats were anesthetized with pentobarbital sodium (30 mg/kg *ip*). A midline laparotomy was performed and the stomach was exposed. The pylorus and cardia were ligated, and the pH electrode of pH/mV meter (59 002-00, Cole-Parmer Instrument Company, USA) was inserted into stomach lumen to measure the pH value of gastric juice[12].

Evaluation of gastric mucosal ulcer index (UI)

The ulcer index (UI) of gastric mucosal lesions was evaluated by the score system reported by Nie SN *et al*[13]. Briefly, after measurement of the pH value of gastric juice, the stomach was opened along the greater curvature and rinsed with 0.1 mol/L ice-cold phosphate-buffered saline (PBS). The stomach was then examined with a 10 × magnifier to observe erosions and make scores as 1-5: 1: small round hemorrhagic erosion, 2: hemorrhagic erosion < 1 mm, Grade 3: hemorrhagic erosion = 1-2 mm, 4: hemorrhagic erosion = 2-3 mm, 5: hemorrhagic erosion > 4 mm. The score was multiplied by 2 when the width of erosion was larger than 1 mm.

H+, K+-ATPase assay

The oxyntic mucosa was scraped with glass slides, immediately frozen in liquid nitrogen and stored at -80 ℃ for H^+, K^+-ATPase assay, at which time they were thawed at 4℃. Microsomal gastric H^+, K^+-ATPase was prepared after homogenization of gastric mucosa and sucrose-Ficoll gradient centrifugation[14,15]. H^+, K^+-ATPase was assessed with a kit (Nanjing Jiancheng Biotechnology Company), and protein was quantified using a UV/spectrophotometer (PE)[16]. The gastric mucosal H^+, K^+-ATPase activity was expressed as μmol Pi/mg protein/h (U/mg prot).

Electron microscopy

Tissue specimens of the oxyntic mucosa for electron microscopy, were immediately fixed in a solution containing 1% glutaraldehyde and 3% formaldehyde buffered at pH 7.4 with 0.1 mol/L sodium PBS for 2 h at 4℃. Secondary fixation was carried out in 1% osmium tetroxide solution buffered at pH 7.4 with 0.1 mol/L sodium phosphate buffer for 1 h at 4℃. The specimens were then dehydrated in graded acetone solutions and embedded in epoxy resin (Epon 812). Ultrathin sections (60-80 nm) were made for double staining with aqueous uranyl acetate and lead citrate, and observed under transmission electron microscope[17]. Parietal cells were recognized by the presence of secretory canaliculi and/or tubulovesicles. Secreting parietal cells were defined as cells having secretory canaliculi. Non-secreting cells had tubulovesicles but no secretory canaliculi[18].

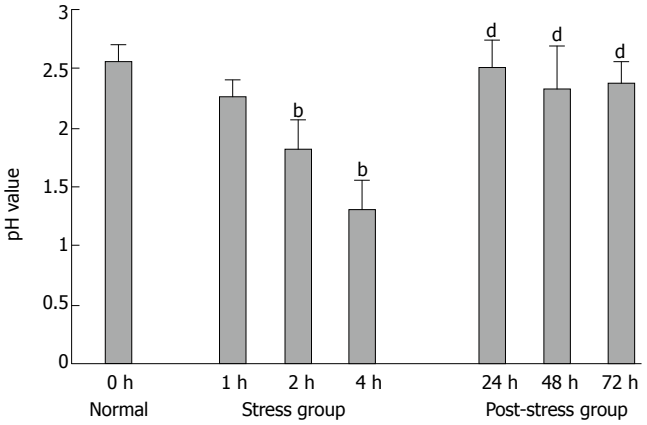


Figure 1 pH value of gastric juice in all groups. $^bP < 0.01$ *vs* normal group; $^dP < 0.01$ *vs* stress 4 h group.

Statistical analysis

All data were expressed as mean ± SD. The statistical differences between different groups were analyzed by Student's *t*-test. The relationship between two variants was analyzed by linearly relevant analysis. $P < 0.05$ was considered significant.

RESULTS

Change of gastric juice pH value

The pH value of gastric juice was 2.56 ± 0.14 in normal group and gradually declined after 1, 2 and 4 h of WRS in rats (2.26 ± 0.04, 1.81 ± 0.25 and 1.31 ± 0.24, respectively) (Figure 1). The pH value was significantly lower (2.0-fold) in stress 4 h group than that in normal group ($P < 0.001$). The pH values of gastric juice after 24, 48, 72 h of WRS in post-stress groups was significantly higher (1.9-fold, 1.8-fold and 1.8-fold, respectively) than that in stress group ($P < 0.001$), but there was no statistical significance compared to normal group ($P > 0.05$, Figure 1). These data demonstrated that the pH value of gastric juice decreased time-dependently in stress group and increased in post-stress group.

Change of gastric mucosal ulcer index (UI)

There was no gastric mucosal lesion in normal group. Scattered spot or lineal erosions, hemorrhage and ulcers were observed in oxyntic mucosa in stress group. The gastric mucosal UI gradually increased after 1, 2 and 4 h of WRS in rats (9.5 ± 2.98, 22.5 ± 3.16 and 35.0 ± 3.93) (Figure 2). The UI decreased significantly (2.0-fold and 4.7-fold) after

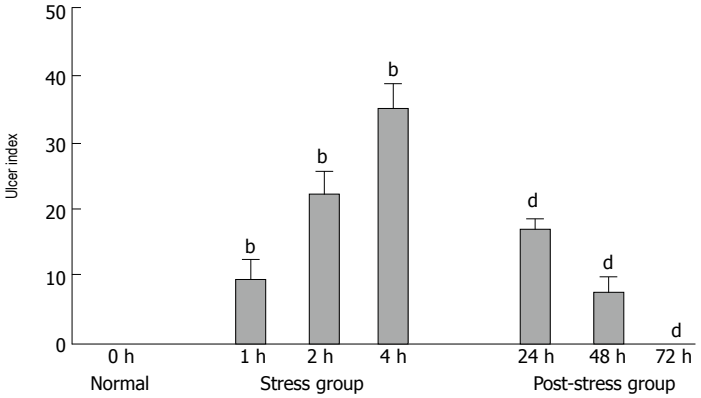


Figure 2 Ulcer index (UI) of gastric mucosa in all groups. [b]$P < 0.01$ *vs* normal group; [d]$P < 0.01$ *vs* stress 4 h group.

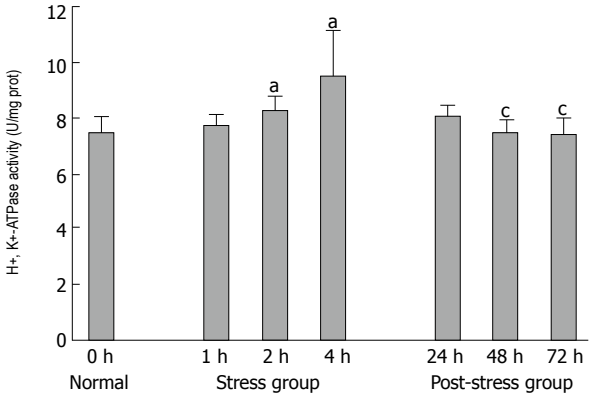


Figure 3 H^+, K^+-ATPase activity of parietal cells in all groups. [a]$P < 0.05$ *vs* normal group; [c]$P < 0.05$ *vs* stress 4 h group.

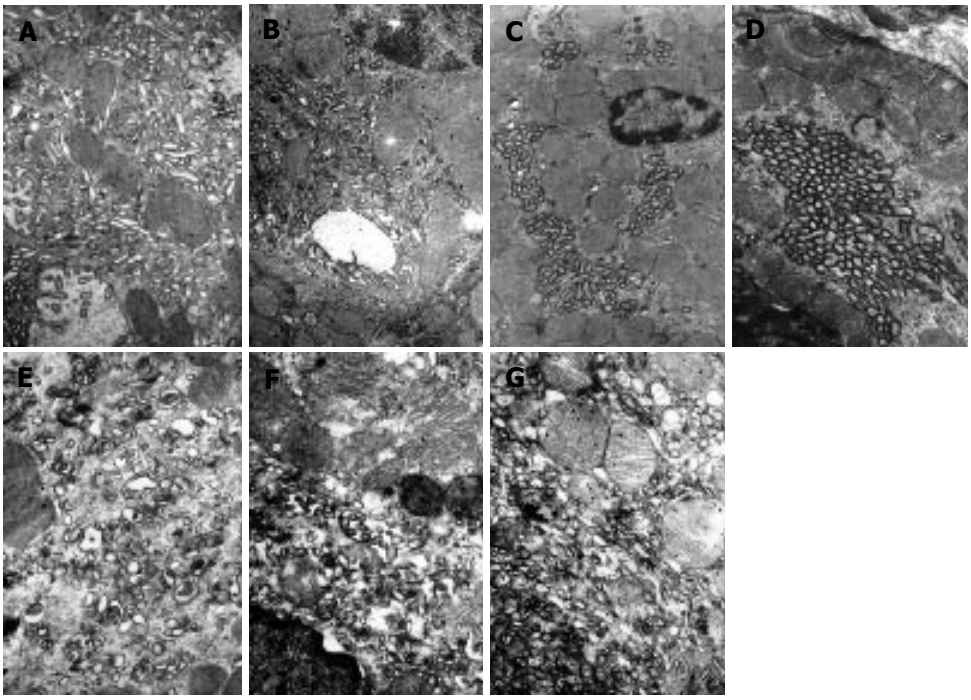


Figure 4 Ultrastructural change of parietal cells. **A:** Gastric parietal cells presented resting state, abundant tubulovesicles and few intracellular canaliculi lined with rare microvilli could be found in the cytoplasm in normal group (×12 000); **B**: Parietal cells presented pre-secreting state, intracellular canaliculi lined with short microvilli were observed, plenty of vesicles were located around the canaliculi and accumulated on the apical plasma membrane in 1 h WRS group (×12 000); **C:** Parietal cells presented secreting state with decreased vesicles and increased intracellular canaliculi lined with elongated microvilli in 2 h WRS group; **D:** Parietal cells presented active secreting state and cytoplasm was almost devoid of vesicles and intracellular canalicular lumen contained numerous elongated microvilli in 4 h WRS group (×12 000); **E - G:** at 24, 48 and 72 h at the end of WRS for 4 h, Parietal cells returned to resting state with abundant tubulovesicles and few canaliculi in cytoplasm in post-stress group swollen mitochondria, vacuolar degeneration, crista-fragmentation, concentrated karyotin, roughness of nuclear envelopes and dilated perinuclear space were observed (×15 000).

24 and 48 h of WRS in post-stress group compared to that in stress group ($P < 0.001$). No lesion occurred in the oxyntic mucosa of rats 72 h after WRS (Figure 2). These data demonstrated that the UI of gastric mucosa increased time-dependently in stress group and decreased in post-stress group. UI was positively related with stress time ($r = 0.9876$, $P < 0.01$) but negatively with pH value ($r = -0.8724$, $P < 0.05$).

H+, K+-ATPase activity

The H^+, K^+-ATPase activity of gastric parietal cells was 7.48 ± 0.59 U/mg prot in normal group and gradually increased after 1, 2 and 4 h of WRS in rats (7.72 ± 0.41, 8.28 ± 0.52 and 9.50 ± 1.63 U/mg prot, respectively) (Figure 3). The H^+, K^+-ATPase activity was significantly higher (1.1-fold and 1.3-fold) in stress group than in normal group ($P < 0.05$). The H^+, K^+-ATPase activity decreased in post-stress group, but there was no statistical significance ($P > 0.05$). The H^+, K^+-ATPase activity was significantly lower (1.3-fold, respectively) after 48 and 72 h of WRS in post stress group than in stress group ($P < 0.05$, Figure 3). These data demonstrated that the H^+, K^+-ATPase activity of gastric parietal cells increased time-dependently in stress group and decreased in post-stress group.

Ultrastructural change of parietal cells

As shown in Figure 4A, gastric parietal cells in normal group presented resting state, a large number of tubulovesicles and few intracellular canaliculi lined with rare microvilli could be found in cytoplasm. As shown in Figure 4B, parietal cells in 1 h WRS subgroup presented pre-secreting state, intracellular canaliculi lined with short microvilli were observed, and plenty of vesicles in the cytoplasm were located around the canaliculi and accumulated on the apical plasma membrane, and the secretory surface was more elaborate than that of the non-secreting cells. As shown in Figure 4C, parietal cells in 2 h WRS subgroup presented secreting state, where decreased vesicles and increased

intracellular canaliculi lined with elongated microvilli were observed. The remaining vesicles were in the region adjacent to the surface and frequently fused with the plasma membrane. In addition, there were abundant mitochondria in the cytoplasm. As shown in Figure 4D, parietal cells in 4 h WRS subgroup presented active secreting state. The cytoplasm was almost devoid of vesicles, whereas the intracellular canalicular lumen contained numerous elongated microvilli. The cytoplasm in these secreting cells was extremely dense owing to the close packing of mitochondria. As shown in Figure 4E-G, in 24, 48 and 72 h WRS subgroups, the parietal cells returned to resting state, abundant tubulovesicles and few canaliculi were observed in the cytoplasm in post-stress group after 24, 48 and 72 h WRS. Besides, Swollen mitochondria, vacuolar degeneration and crista-fragmentation were observed in some parietal cells. Concentrated karyotin, roughness of nuclear envelopes and dilated perinuclear space were observed in other cells, which might be due to the damaged parietal cells induced by stress.

DISCUSSION

Stress ulcers are the superficial mucosal lesions located predominantly in fundus of the stomach. They occur mainly in severe trauma and sepsis as well as in acute severe psychological stress. It has become apparent that the physiological stress of critical illness increases the risk of developing stress-related mucosal injury and gastrointestinal bleeding. The pathogenesis of stress ulcers is not completely understood. however, recent studies indicate that the origin may be multifactorial, and there is an imbalance between protective and destructive factors[19-21]. Major destructive factors include acid, pepsin, bile, reperfusion injury and free oxygen radicals[22-24]. Protective factors include adequate mucosal blood flow, mucus-bicarbonate layer, epithelial cell renewal and prostaglandins[25-27]. It is generally agreed that mucosal ischemia is the major inciting event in the pathogenesis of acute stress ulceration of the stomach and that the presence of luminal acid and pepsin is required in the development of overt ulceration[28]. Back-diffusion of acid occurs in the absence of overt disruption of the mucosal barrier, and is closely related to the formation of ulcers.

In this study, we found that gastric mucosal lesions gradually aggravated after 1, 2 and 4 h of WRS in rats. Gastric mucosa lesions progressed to apparent erosions and hemorrhage in 4 h WRS group. There positive relevance between the ulcer index UI was significantly related with the exposure time to WRS. We also found that gastric juice pH value decreased and H^+, K^+-ATPase activity of parietal cells gradually increased after 1, 2 and 4 h of WRS, indicating that gastric acid secretion increases with the exposure time to WRS. The pH value was significantly lower (2.0-fold) and the H^+, K^+-ATPase activity was significantly higher (1.3-fold) in stress group than in normal group. UI was negatively related with pH value. These results strongly suggest that gastric acid can mediate acute gastric mucosal lesions induced by WRS.

The present study assessed the dynamic ultrastructural change of gastric parietal cells in rats induced by WRS. Gastric parietal cells also known as oxyntic cells, have two systems in the cytoplasm under transmission electron microscope: intracellular secretory canaliculus system and tubulovesicular system[29]. In normal group, abundant tubulovesicles and few intracellular canaliculi lined with rare microvilli were found in cytoplasm and the gastric parietal cells presented resting state. Tubulovesicles gradually decreased while intracellular canaliculi lined with elongated microvilli gradually increased after exposed to WRS for 1, 2 and 4 h. In 4 h WRS group, parietal cell cytoplasm was almost devoid of vesicles and the intracellular canalicular lumen contained numerous elongated microvilli. Our observation showed that parietal cells presented a series of states from resting, pre-secreting, secreting to active secreting after 0, 1, 2 and 4 h of WRS. Our study also showed that gastric mucosal lesions induced by WRS paralleled to time-course changes of ultrastructure of gastric parietal cells. These results indicate that WRS can time-dependently activate parietal cells and induce gastric acid secretion.

The mechanism of the recovery of gastric mucosa after stress is not fully understood. the healing of stress ulcer is a very complex process affected by multi-factors. It has been reported that the mucosal integrity and repair involved enhancement of gastric mucosal blood flow and increased mucosal cell proliferation mediated by epidermal growth factor (EGF) and transforming growth factor alpha (TGFα)[30,31]. The importance of gastric acid in the process of restructuring gastric mucosa after WRS has not yet been evaluated. In present study, we found that the injured gastric mucosa was gradually restructured after the stress was removed and no lesion was observed after 72 h at the end of WRS for 4 h. We also found the pH value increased significantly at 24, 48 and 72 h of WRS. These results strongly demonstrate that the decrease of gastric acid is beneficial for restructuring injured gastric mucosa.

In the present study, we found that the parietal cells returned to resting state while swollen mitochondria, vacuolar degeneration, crista-fragmentation, concentrated karyotin, roughness of nuclear envelopes and dilated perinuclear space were observed after 24, 48 and 72 h of WRS, suggesting that damaged parietal cells induced by WRS.

The major founding of our study is that the ultrastructural changes of parietal cells is consisitent with the change of H^+, K^+-ATPase activity in the development and healing of stress ulceration induced by WRS. Moreover, the recovery of ultrastructural and functional changes of parietal cells was earlier than the restructuring of damaged gastric mucosa after WRS, suggesting that the ultrastructural and functional changes of parietal cells may be an important pathophysiologic mechanism in restructuring stress ulceration.

In conclusion, ultrastructural and functional changes of gastric parietal cells are induced by WRS in rats. Increased gastric acid secretion induced by WRS is consistent with the ultrastructural changes of parietal cells and is significantly correlated with the degree of gastric mucosa lesions. Gastric acid not only plays an important role in the development of stress ulcer, but also is closely related to the recovery of gastric mucosal lesions induced by WRS.

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BASIC RESEARCH

Cloning and molecular characterization of Δ^{12}-fatty acid desaturase gene from *Mortierella isabellina*

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Abstract

AIM: To clone Δ^{12} -fatty acid desaturase gene of *Mortierella isabellina* and to functionally characterize this gene *in vitro* and *in vivo*.

METHODS: Reverse transcriptional polymerase chain reaction (RT-PCR) was used to clone the open reading frame of Δ^{12}-fatty acid desaturase gene (D12D) of *Mortierella isabellina*. Plasmids pEMICL12 and pYMICL12 were constructed with it. pEMICL12 was transformed into *Escherichia coli* (*E.coli*) strain BL21 using $CaCl_2$ method for expression after induction with IPTG. pTMICL12 was transformed into *Saccharomyces cerevisiae* strain INVSc1 using lithium acetate method for expression under the induction of galactose. Northern blotting method was used to investigate the effect of temperature on the transcriptional level of this gene in *S.cerevisiae* strain INVSc1.

RESULTS: Recombinant plasmids pEMICL12 and pTMICL12 were successfully constructed and transformed into *E.coli* and *S.cerevisiae* separately with appropriate method. After induction with IPTG and galactose, it was found that expression of Δ^{12}-fatty acid desaturase genes in *E.coli* and *S. cerevisiae* under appropriate conditions led to the production of active Δ^{12}-fatty acid desaturase, which could convert 17.876% and 17.604% of oleic acid respectively to linoleic acid by GC-MS detection *in vitro* and *in vivo*.

CONCLUSION: Cloning and expression of *M.isabellina* D12D gene in *E.coli* and *S.cerevisiae* is successfully completed.

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Key words: *Mortierella isabellina*; Δ^{12}-fatty acid desaturase; *In vitro* expression

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INTRODUCTION

As an economically important filamentous fungi, *M isabellina* which belongs to *Mucor sp.* can produce linolenic acid(GLA) that plays very important roles in regulating the hormone level and fatty acid metabolism in human. In addition, recent study indicated that GLA can alleviate the symptoms of many kinds of diseases such as neurodegeneration[1,2] , adipogenesis[3], cancer[4,5] , Protofibrils[6-8] , cardiovascular disease[9-11], diabetes[12] and psoriasis[13]. Being a kind of nutritionally and pharmaceutically important substance, GLA has become the hotspot both in academic and applied fields. *M isabellina* has been a widely used strain in industry to produce GLA by fermentation, in which GLA accounts for 8 percent of total fatty acids. Further efforts to enhance the percentage of the GLA by traditional mutation or by changing fermentation conditions such as medium and temperature have been made, but the extent is relatively small. Therefore, we attempted to reconstruct this strain with genetic engineering.

In most eukaryotic cells, GLA is biosynthesized by two desaturation processes, using stearic acid C18:1 as initial substrate[14-17] . Linoleic acid (LA) C18:2 is synthesized from C18:1 by the reaction catalyzed by Δ^{12}-fatty acid desaturase and then LA can be converted to GLA by Δ^{6}-fatty acid desaturase. LA can also be converted to α-linolenic acid (ALA) by ω3 fatty acid desaturase. LA and ALA are so-called essential fatty acids (EFAs)[18,19] to human bodies because of their inability to synthesize these compounds *de novo*. Linoleic acid is the precursor of polyunsaturated fatty acids (PUFAs) in both routes of n-6 and n-3[20]. Therefore, Δ^{12}-fatty acid desaturase is the key enzyme for producing LA and is also the speed-limiting enzyme for routes of n-6 and n-3. Cloning and characterization of Δ^{12}-fatty acid desaturase gene will help understand the biosynthetic pathway of GLA and further enhance the percentage of GLA in total fatty acids.

Our research interest focuses on PUFAs having 18

carbon atoms or more such as linoleic acid and linolenic acid in fungus. In this paper, we report the isolation of cDNA of D12D from *M isabellina*. The cDNA was expressed in a classical system, *S.cerevisiae* and *E.coli*.

MATERIALS AND METHODS

Materials and culture conditions

Mortierella isabellina M_{6-22} was grown at 28℃ for 2 d in liquid medium containing 2% glucose, 1% bacto-yeast extract, 0.2% KH_2PO4 and 0.1% $MgSO_4$, with pH adjusted to 6.0. *S. cerevisae* strain INVScI was used as recipient strain in transformation experiments and was grown at 30℃ in complex medium containing 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose. *E.coli* strain DH5a was grown at 37℃ in Luria–Bertani medium (LB) supplemented with 100 mg/L of ampicillin. *E.coli* strain BL21 and the expression vector pET21a were purchased from Novegon(Novegen Inc., Madison, WI).

Restriction endonucleases, and the other DNA-modifying enzymes were obtained from TaKaRa Bio. Dalian, China Co. Ltd. Other chemicals were purchased from Sangon Bio. Shanghai, China Co. Ltd.

RNA extraction

Mycelia were harvested by filtration and washed with phosphate buffer. The extraction of total RNA was done according to the guanidinium thiocyanate method as described by Chomczynski and Sacchi[21] and extracts were stored at -70℃ for future use.

Cloning of Δ^{12}-fatty acid desaturase gene by RT-PCR

A pair of primers was designed according to the reported sequence of Δ^{12}-fatty acid desaturase gene from *Mortierella alpine*[22] to clone the open reading frame (ORF) of the *M isabellina* D12D gene, P1: 5'-TAC CTC ***GAA TCC*** **ATG** GCA CCT CCC AAC ACT ATC GAT GCC-3'; P2: 5'-AAC CGT ***CTC GAG*** **TTA** CTT CTT GAA AAA GAC CAC GTC TCC-3', which corresponded to the nucleotide sequences of start and stop codons (in boldface) of the Δ^{12}-fatty acid desaturase gene, respectively. The 5'-end of both primers contained an *Eco*R Ⅰ and an *Xho* Ⅰ site(underlined and in boldface), respectively that were underlined to facilitate subsequent manipulation. PCR was performed in a Biometra T-gradient thermal cycler using the following program: at 94℃ for 1 min, 53℃ for 1 min, 72℃ for 2 min for 30 cycles, followed by extension for 10 min at 72℃. PCR fragments were subcloned into pGEM-T vector (Promega, Madison, WI) to produce recombinant plasmid pTMICL12, which was then transformed into *E.coli* DH5a. Subsequently, nucleotide sequences were determined (TaKaRa Bio, Dalian, China). Analysis of the sequences was done with DNAMAN software (version 4.0, Lynnon BioSoft, Quebec).

Plasmid construction

The ORF of Δ^{12}-fatty acid desaturase from *M isabellina* was digested from pTMICL12 with *Eco*R Ⅰ and *Xho* Ⅰ and subcloned respectively into *S.cerevisiae* expression vector pYES2.0 and *E.coli* expression vector pET21a. Positive transformants were screened on the LB plate with ampicillin resistance. The resultant positive plasmid was validated by PCR and restriction enzyme digestion. The construction sketch of pYMICL12 is shown in Figure 1. The resultant plasmids, pYMICL12 and pEMICL12 were stored and propagated in *E.coli* strain DH5a.

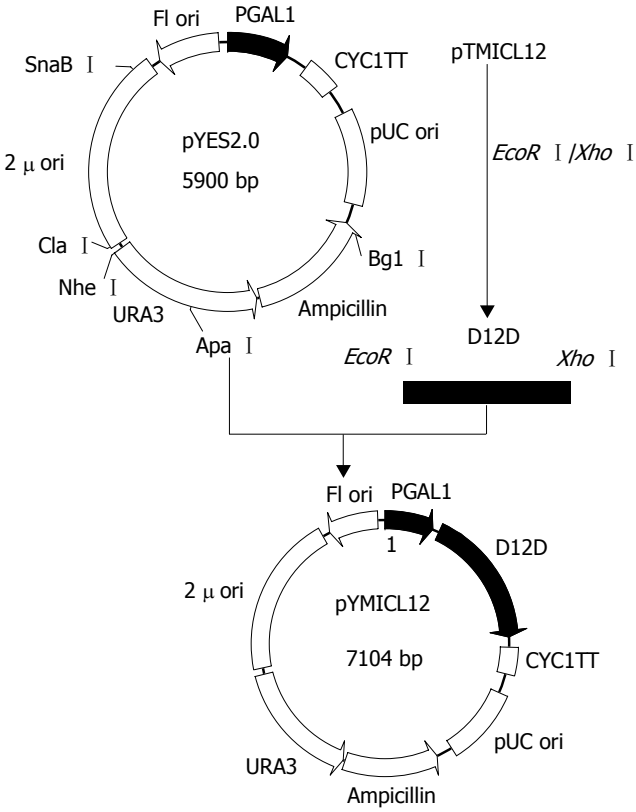


Figure 1 Construction sketch of pYMICL12.

Yeast transformation and inducement of transgenic yeast

The recombinant plasmid pYMICL12 was transformed into *S.cerevisiae* INVSc1 using the lithium acetate method [23]. Positive transformants were selected by plating on complex synthetic minimal medium agar lacking uracil (SC-Ura) and grown at 30℃ for 2-3 d. The putative Δ^{12}-desaturase gene was heterologously expressed in yeast, which was induced under transcriptional control of the GAL1 promoter. Yeast cultures were grown to logarithmic phase at 30℃ in synthetic minimal medium containing different concentrations of galactose(1%, 2%, 3%, 4% respectively), and 0.67% yeast nitrogen. Subsequently, cells were harvested by centrifugation followed by washing in sterile water for three times. The cells were dried and ground into fine powders for determination of fatty acid composition by gas chromatography and for gas chromatography–mass spectrometry (GC-MS) analysis.

Inducement of the transgenic E.coli cells

Transformant containing plasmid pETMICL12 was cultured in M9 medium at 37℃ overnight. Five hundred milliliter M9 medium was inoculated into 2.8 L Fernbach flask with aliquot from the overnight culture until the light density reached 0.5. IPTG was added until the final concentration reached 0.4 mmol/L. After 4 hours' induction,

cells were pelleted by centrifugation at 5000 r/min for 10 min at 4℃ and stored at -70℃ for future use.

Preparation of cell membrane

Cells defrosted at room temperature were suspended in 30 mL buffer A (containing 10 mmol/L $MgCl_2$, 50 mmol/L MOPS-NaOH, 1 mmol/L EGTA, 10 μg/mL DNase) and then were treated with supersonic 450 Hz for 40 cycles, 4 s per cycle. Intact cells and cell debris were pelleted by centrifugation at 5000 r/min for 10 min at 4℃. Supernatants containing cell membrane were pelleted by super-centrifugation at 150 000 r/min for 30 min at 4℃ and then were re-suspended in 200 μL buffer A.

SDS-PAGE

Ten percent of the gel was used to separate the protein bands, and stained by Coomassie Blue R-250[24].

In vitro assay of activity of Δ^{12}-fatty acid desaturase

Sodium salt was added into the membrane suspension in buffer A to reach the final concentration of 150 μmol/L and then the mixture was incubated at 25℃ in water bath for 30 min after oleic acid was added. Soon after that, the mixture was extracted with chloroform methanol and water (volume ratio 2:1:2). The extraction was methyl-esterified with 5% HCl/methanol at 85℃ for 150 min. Fatty acid methyl ester was solubilized with hexane[25,26].

Detection of desaturase activity expressed in transgenic yeast

Total fatty acid was extracted from the cells by treating 100 mg yeast powder with 5 mL 5% KOH in methanol for saponification at 70℃ for 5 h. pH of the product was adjusted to 2.0 with HCl (6 mol/L) before the fatty acid was methyl-esterified with 4 mL 14% boron trifluoride in methanol at 70℃ for 1.5 h. Then, fatty acid methyl esters (FAME) were solubilized with hexane after addition of saturated sodium chloride solution. FAMEs were analyzed by gas chromatography (GC; GC-9A, Shimadzu, Kyoto, Japan) and identified by the comparison of their peaks with that of standards (Sigma). Qualitative analysis of FAME was performed by GCMS using an HP G1800A GCD system (Hewlett–Packard, Palo Alto, CA, USA). Both analyses were carried out with the same polar capillary column (HP, 5.30 mU, 0.25 mm in internal diameter). The mass spectrum of a new peak was compared with that of the standard for identification of fatty acid.

Analysis of mRNA level in transgenic yeast after induction at different temperature

Northern blotting was used to investigate the effect of temperature on the transcriptional level of MID12. Total RNA of transgenic yeast after induction at different temperatures was extracted by UNIQ-10 column (Sangon Corp.) and digoxingenin (Roche Diagnostics Corp.) was used to label the DNA probe (PCR of MID12)

RESULTS

Target gene obtained

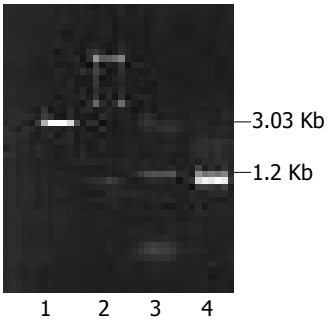


Figure 2 Identification of the recombinant plasmid, pTMICL12 by restriction enzyme. 1: pGEM-T/*Eco*R Ⅰ/*Xho* Ⅰ; 2: DNA size marker λ DNA/*Bst* Ⅱ; 3: pTMICL12/*Eco*R Ⅰ/*Xho* Ⅰ; 4: PCR product of D12D DNA.

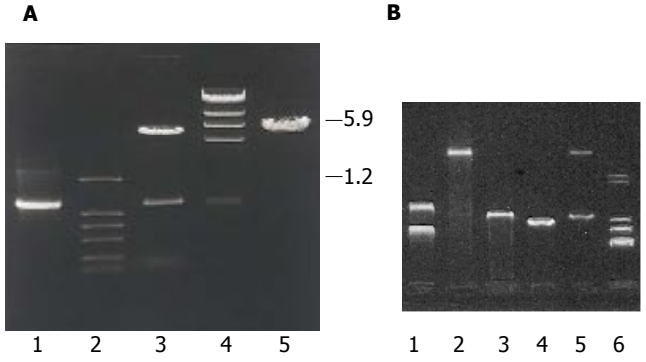


Figure 3 **A**: Identification of pYMICL12. 1: PCR product of D12D DNA; 2: DNA size marker DL2000; 3: pYMID12/*Eco*R Ⅰ/*Xho* Ⅰ; 4: DNA size marker λDNA/Hind Ⅲ; 5: pYES2.0/*Eco*R Ⅰ/*Xho* Ⅰ. **B**: Restriction analysis of recombinant plasmids pEMICL12. 1: Plasmid pEMICL12; 2: PCR product of D12D; 3: pET21a/ *Eco*R Ⅰ; 4: pMAGL12/ *Xho* Ⅰ; 5: pEMICL12/ *Eco*R Ⅰ + *Xho* Ⅰ; 6: λDNA/HindⅢ marker.

PCR product about 1.2 kb long was gel-purified by electrophoresis and cloned into pGEM-T vector. Positive clones were selected on LB plate by color reaction. PCR and enzyme restriction were used to analyze the resultant transformant named pTMICL12. After digestion with *Sac*Ⅱ and *Pst* Ⅰ, a 3.03 kb fragment and a 1.2 kb target gene were obtained as shown in Figure 2. Subsequently, nucleotide sequences were determined (TaKaRa Bio, Dalian, China)

Nucleic acid sequence of Δ^{12} -fatty acid desaturase in *M. isabellina* has been deposited in Genbank database and assigned the accession No. AF417245. Comparison of the DNA sequence with other Δ^{12}-fatty acid desaturase gene showed that it has the highest identity with Δ^{12}-fatty acid desaturase gene from *M. alpina* (about 99.92%) and higher identity with other D12D from fungi[27-30] (more than 40%). Therefore, this sequence encoded a putative Δ^{12}-fatty acid desaturase gene.

Expression vector construction for S. cerevisiae and E coli

In order to investigate the function of this sequence, target gene was subcloned into YES2.0 and pET21a to construct two recombinant plasmids pYMICL12 and pEMICL12 which were transformed into *E.coli* DH5a for propagation. After digested by *Eco*R Ⅰ and *Xho* Ⅰ, both of the plasmids released a 1.2 kb fragment (Figure 3), indicating that the recombinant plasmids were successfully reconstructed.

Detection of desaturase activity in transgenic yeast

After inducement, the total fatty acid in transgenic yeast

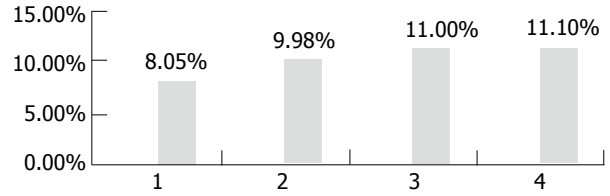


Figure 4 Percentage of linoleic acid in transgenic yeast induced by different concentration of galactose. 1: 1% galactose; 2: 2% galactose; 3: 3% galactose; 4: 4% galactose.

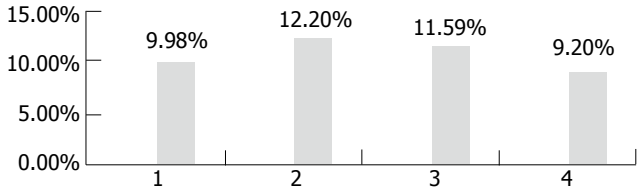


Figure 5 Percentage of linoleic acid in transgenic yeast induced for different durations. 1.12 h; 2. 24 h; 3.36 h; 4. 48 h.

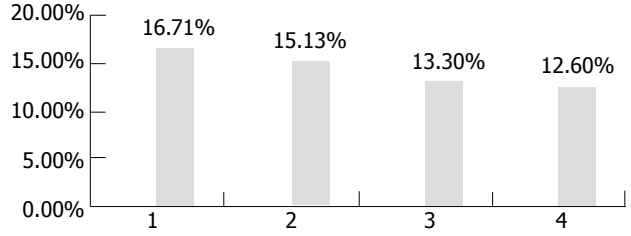


Figure 6 Percentage of linoleic acid in transgenic yeast induced at different temperature. 1: 15℃; 2: 20℃; 3: 25℃; 4: 30℃.

harboring plasmid pYMICL12 was extracted and analyzed by GC. It was found that under different conditions, the expressed desaturase showed different activities.

First, the transgenic yeast was induced at 30℃ for 36 h, with different concentrations of galactose: 1%, 2%, 3%, 4%. The result of GC showed that percentage of LA in transgenic yeast was the highest when it was induced with 3% galactose (Figure 4).

Then, different duration times were used to induce the transgenic yeast by 3% galactose at 30℃ for 12, 24, 36, and 48 h respectively. The result of GC showed that the optimal induction time was 24 h as shown in Figure 5.

Thirdly, the transgenic yeasts were induced by 3% galactose for 24 h at different temperatures of 15℃, 20℃, 25℃, and 30℃ respectively. It was found that the lower the temperature, the higher the activity of the expressed enzyme. The highest percentage of LA reached 16.706% of total fatty acid in induced yeast harboring plasmid pYMICL12 when the temperature was 15℃ (Figure 6).

Therefore, the optimal condition for expressing D12D from *M isabellina* was: 3% galactose induction for 24 h at 15℃. GC analysis of the fatty acid composition of the transgenic yeast containing plasmid pYMICL12 indicated that a novel peak that had the same retention time with that of LA standard appeared. That specific peak was absent from the control transformant (Figure 7).

Northern blot

Northern blot was performed to check if there was an increase of mRNA after induction by low temperature which resulted in the higher activity of Δ^{12}-fatty acid desaturase in transgenic yeast, INVSc1(pYMICL12).

After induction at different temperatures, total RNA was extracted. In the formaldehyde-denaturalization electrophoresis, 30 mg RNA was used for each sample. The result of the Northern blot is shown in Figure 8.

Any visible change of the level of RNA could not be found on the photo of Northern blot. Analysis by VDS-scan also showed no obvious difference between the samples, implying there were no changes of the mRNA level of D12D in the transgenic yeast at different temperature. The level of transcription was not the key cause for the change of LA level in INVSc1 (pYMICL12) under cold condition. The increasing activity of Δ^{12}-fatty acid desaturase might come from the protection of the enzyme activity by low temperature, which even prolonged the half life of Δ^{12}-fatty acid desaturase mRNA.

Detection of Δ^{12}-fatty acid desaturase expressed in E coli by SDS-PAGE

Transformants harboring plasmid pEMICL12 were cultured in M9 medium and induced by IPTG to express Δ^{12}-fatty acid desaturase from *M isabellina*. Cell membranes were pelleted by super-centrifugation. Analysis with SDS-PAGE showed that a specific protein band that had the molecular weight of approximately 43 kd existed on the gel, whereas cell membrane from control transformant harboring the plasmid pET21a did not express the specific protein. In addition, the molecular weight of the specific protein band was nearly identical to other reported Δ^{12}-fatty acid desaturase. These results suggested that this specific band putatively was Δ^{12}-fatty acid desaturase (Figure 9).

In vitro assay of activity of Δ^{12}-fatty acid desaturase

In order to test whether this protein band could convert OA to LA, *in vitro* assay with membrane protein was done. *E coli* can not produce OA that is the most commonly used substrate of Δ^{12}-fatty acid desaturase. Therefore, OA must be added into the reaction mixture to be converted to LA by Δ^{12}-fatty acid desaturase. GC analysis of the mixture after reaction indicated that transformant containing plasmid pETMICL12 exhibited Δ^{12}-fatty acid desaturase activity and that the membrane protein could convert 17.604% of the OA to LA(indicated by arrow), whereas cell membrane protein from transformant containing empty plasmid pET21a did exhibit Δ^{12}-fatty acid desaturase activity. Also, the retention time of the specific peak in Figure 10 was identical to standard LA from Sigma. These results indicated that the specific band had Δ^{12}-fatty acid desaturase activity.

DISCUSSION

M isabellina strain $M_{6\text{-}22}$ is a mutant strain in which total fatty acids account for 60 percent of the dry weight of the mycelium. Also, it synthesizes PUFAs only up to 18-carbon with c isomer of linolenic acid (GLA), which make the subsequent purification relatively easy. Thus, *M isabellina*

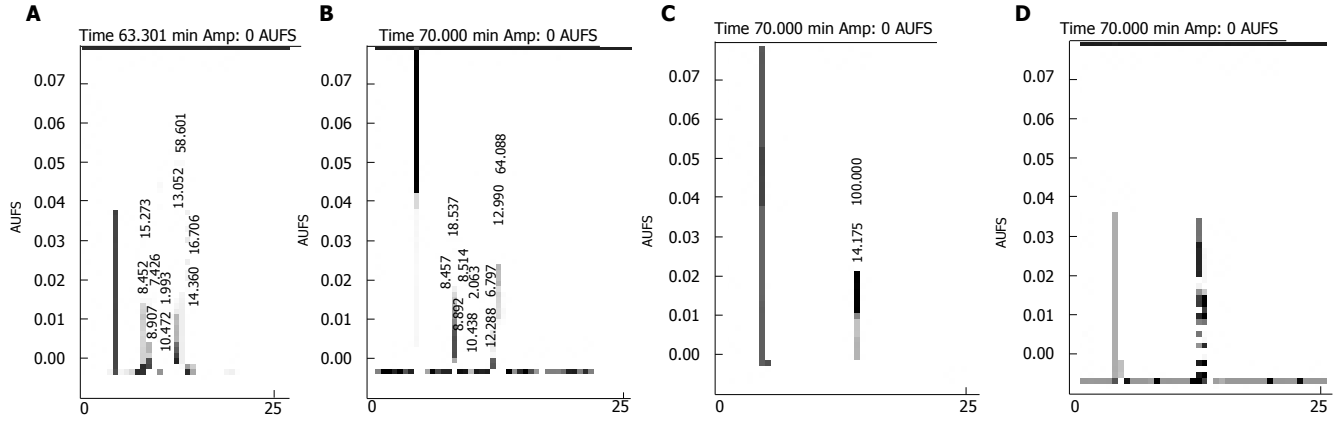


Figure 7 GC analysis of the total fatty acid in transgenic yeast, INVSc1 (pYMICL12). **A**: Total fatty acid in transgenic yeast, INVSc1 (pYMICL12) after induction by 3% galactose at 15℃ for 24 h; **B**: Total fatty acid in transgenic yeast, INVSc1 (pYES2.0) after induction by 3% galactose at 15℃ for 24 h; **C**: Linoleic acid; **D**: Oleic acid.

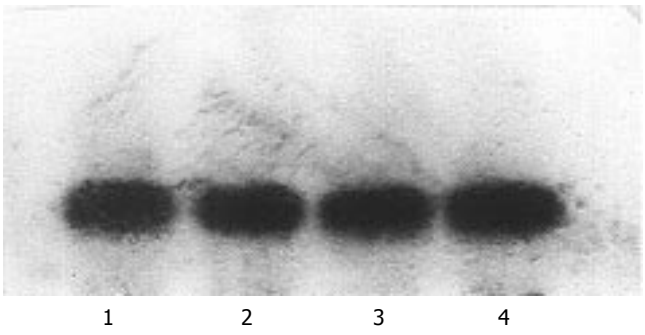


Figure 8 Northern blot of total RNA of transgenic yeast induced at different temperature. 1: 15℃; 2: 20℃; 3: 25℃; 4: 30℃.

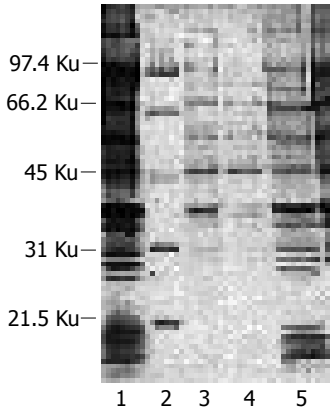


Figure 9 SDS-PAGE of membrane protein in transgenic *E.coli*.1.Total protein of BL21; 2: low mass marker; 3: Total protein of BL21/pMICL12; 4: Total soluble protein of BL21/pMICL12 after supercentrifugation; 5: Membrane protein of BL21/pMICL12.

strain $M_{6\text{-}22}$ is a promising producer of GLA and also a potential strain to be used to produce other nutritionally and pharmaceutically important PUFAs. However, increasing the percentage of GLA meets great challenges, which made us to turn to other methods including genetic engineering. As we all know, cloning the genes encoding corresponding enzymes is the first thing we should do. In our previous work, we have successfully cloned and characterized Δ^6-fatty acid desaturase gene from *M isabellina*[31]. In order to further elucidate the biosynthetic pathway of polyunsaturated fatty acid, we decided to clone the enzyme genes that are ahead of the Δ^6-fatty acid desaturase gene in reaction chains.

As *M isabellina* is very near to *M alpine* in phylogeny, we adopted the strategy of direct cloning the gene by designing the primer identical to the reported *M alpine* Δ^{12}-fatty acid desaturase gene. Using this method, a fragment of proximately 1.2 kb was obtained. Sequence comparison indicated it has the highest identity with *M alpine* Δ^{12}-fatty acid desaturase gene. In order to functionally identify this nucleic acid sequence, two expression systems were applied: yeast and *E.coli*. In both systems, the heterologously expressed proteins exhibited Δ^{12}-fatty acid desaturase activity, indicating that this sequence encoded a functional desaturase. The high identity between the two sequences suggests that *M alpine* and *M isabellina* belong to the same species. But there indeed exist great differences between the two species. *M alpine* can produce many kinds of PUFAs, but *M isabellina* can only synthesize PUFAs up to 18-carbon. This indicates that other factors may contribute to the difference.

Protein expression in yeast is influenced by many factors such as temperature, induction time. In this work, different temperatures were used. In a certain extent (15-30℃), the higher the temperature, the lower the desaturase activity. At even lower temperature, the yeast INVSc1 (pYMICL12) could achieve more LA. However, a temperature lower than 15℃ would hinder the growth of the mycelium, which reduced the total activity of Δ^{12}-fatty acid desaturase.

Although desaturase activity increased as the temperature decreased, northern blotting analysis indicated that the increasing of desaturase activity was not due to the corresponding increase of the mRNA level. In our study, no significant difference existed among the mRNA level at different temperatures. Other researchers also have reported the same result[28]. This indicates that under certain conditions, mRNA level can not be influenced by temperature. The most important factor that can change it may be the promoter activity. Does the increased activity of Δ^{12}-fatty acid desaturase, however, come from the influence of coldness on mRNA level or protein level? Or does longer half life of Δ^{12}-fatty acid desaturase even directly influence activity of protein? There are different opinions about this in academia.

In conclusion, this report about cloning and character-

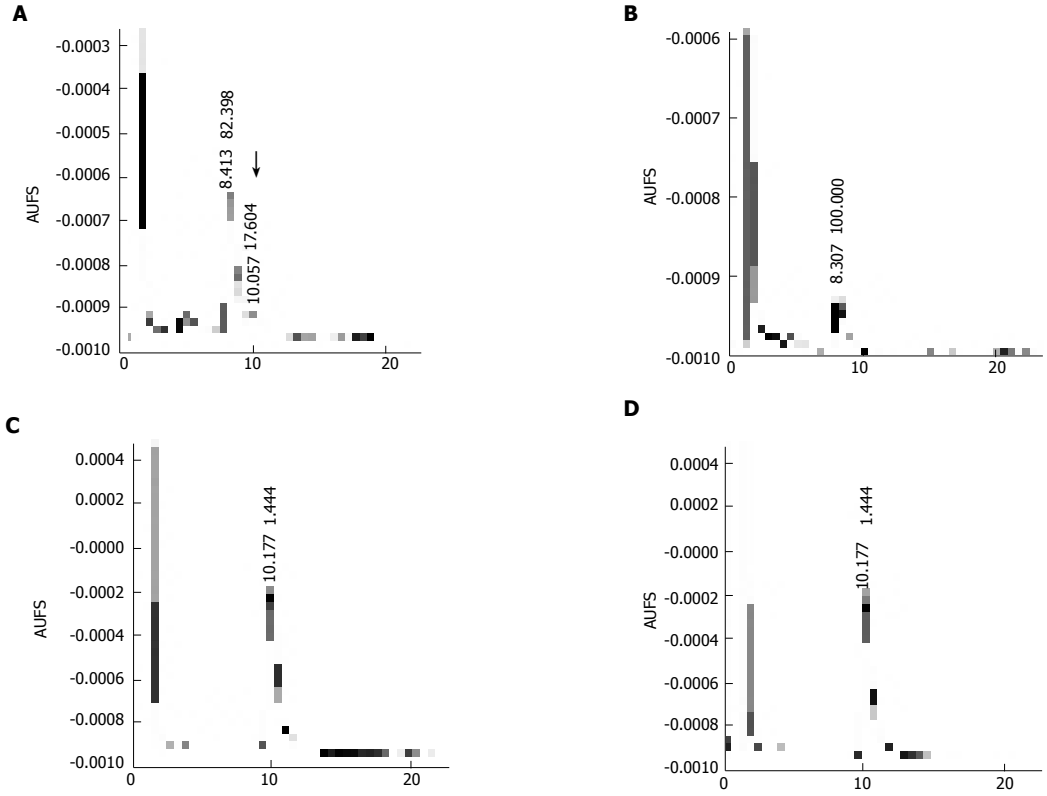


Figure 10 Gas chromatographic analysis of fatty acid methyl esters of lipids catalysed by membrane protein of *E.coli* in system *in vitro*. **A**: Fatty acid components catalysed by membrane protein of BL21/ pMICL12; **B**: Fatty acid components catalysed by membrane protein of BL21/ pET21a; **C**: Linoleic Acid; **D**: Oleic Acid.

ization of the *M isabellina* Δ^{12}-fatty acid desaturase gene will be helpful for us to utilize the industrially used strains.

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CLINICAL RESEARCH

Malnutrition affects quality of life in gastroenterology patients

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Abstract

AIM: To investigate the association between malnutrition and quality of life in patients with benign gastrointestinal disease.

METHODS: Two hundred patients (104 wellnourished and 96 malnourished) were assessed according to the Subjective Global Assessment, anthropometric measurements and bioelectrical impedance analysis. Quality of life was determined with the validated Medical Outcomes Study 36-item Short-Form General Health Survey (SF 36). Muscle function was assessed by hand grip strength and peak flow.

RESULTS: Body mass index, body cell mass, arm muscle area and hand grip strength were significantly lower in the malnourished patients. Quality of life was generally lower when compared to norm values. Seven out of eight quality of life scales (excluding *bodily pain*) were significantly reduced in the malnourished patients. Comparing patients with liver cirrhosis and inflammatory bowel disease (IBD), patients with IBD experienced significantly lower values in the perception of *bodily pain,social functioning* and *mental health*. Malnourished liver cirrhotics suffered reductions in more scales (six out of eight) than malnourished IBD patients did (four out of eight).

CONCLUSION: Quality of life is generally low in benign gastrointestinal disease and is further reduced in patients who are classified as malnourished. It appears that liver cirrhosis patients experience a higher quality of life than IBD patients do, but the impact of malnutrition seems to be greater in liver cirrhosis than in IBD.

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Key words: Quality of life; Malnutrition; Gastrointestinal disease; Liver cirrhosis; Inflammatory bowel disease

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INTRODUCTION

Malnutrition is common in chronic and acute gastrointestinal disease affecting both morbidity and mortality. Impairment of nutritional status frequently also decreases muscle function[1,2], which lastly results in an impaired functional status.

Studies have shown a close relationship between malnutrition and decreased quality of life in some populations such as elderly institutionalized patients[3,4], patients with cancer[5-7] and patients on haemodialysis[8-10]. Quality of life is a subjective multidimensional construct reflecting functional status, emotional and social well being as well as general health. Its measurement is based on the patient's perception of well being.

In order to evaluate patients' situation or benefits of new treatment strategies, quality of life has become a new clinically relevant outcome parameter[11-13]. In chronic disease in particular, where recovery from disease is not always achievable, quality of life is essential and its measurement should be integrated as one main intervention target. It is therefore crucial to better understand the relationship between more objective measures such as disease parameters, nutritional status and subjective quality of life.

Studies investigating factors influencing quality of life in benign gastrointestinal (GI) disease have not looked at the impact of nutritional deficiency[14-17]. Hitherto, there are no data on quality of life in relation to nutritional status in patients with benign gastrointestinal disease. Since malnutrition frequently occurs in gastroenterological disease, we explored the impact of malnutrition as identified by the Subjective Global Assessment in patients with benign gastroenterological disease. We also focused the evaluation on subgroups of our study patients with chronic gastrointestinal disease like liver cirrhosis or chronic inflammatory bowel disease (Crohn's disease or ulcerative colitis).

MATERIALS AND METHODS

Patients

A total of 200 patients with benign gastroenterological disease admitted to a gastroenterology ward at our university hospital were recruited for the study. Sixty one patients with liver cirrhosis (n = 33 men and n = 28 women), 69

patients with inflammatory bowel disease (n = 29 men and n = 40 women) as well as 70 patients with various acute and chronic gastroenterological diagnoses (n = 32 men, n = 38 women) were included in the study (Table 1). All patients were considered for inclusion if they had a non-neoplastic disease regardless of disease severity. Exclusion criteria were malignant comorbidity and terminal disease (life expectancy < 3 mo) in order not to bias interpretation of quality of life. Patients with neuro-muscular disease, hemiplegia or rheumatoid arthritis were excluded in order to avoid potential confounders on muscle strength. Furthermore, patients with implanted defibrillator were excluded from the bioelectrical impedance analysis. All measurements were performed within 36 h of admission. Patient recruitment was consecutive until at least 100 well nourished patients with benign gastrointestinal disease were included. Then, only malnourished patients were consecutively included in order to obtain comparable patient groups.

All patients signed written informed consent and this study was approved by the local ethics committee.

Table 1 Clinical diagnoses of the study population

Diagnoses	*n*		*n*		*n*
Liver cirrhosis	**61**	**Others**	**70**		
Alcoholic liver cirrhosis	51	Biliary disease	11	Functional dyspepsia/ IBS	4
Cryptogenic liver cirrhosis	4	Gastritis / reflux disease	11	Ulcer disease	3
Biliary liver cirrhosis	4	Chronic pancreatitis	9	Colon and/or rectal polyps	2
Post hepatic liver cirrhosis	2	Non infectious colitis	7	Malabsorption syndrome	2
Inflammatory bowel disease	**69**	Diverticulitis	8	Viral hepatitis	2
Crohn's disease	46	Fatty liver disease	8	Proctologic disease	2
Ulcerative colitis	23			**Total**	**200**

Quality of life

Quality of life was assessed employing the validated Medical Outcomes Study 36-item Short-Form General Health Survey described in detail elsewhere[18,19]. The questionnaire consists of 36 questions forming 8 multi item scales and is self administered [18,19]. These scales range from 0-100 percent (absolute values), but norm values differ substantially across the scales. In order to facilitate interpretation and to compare the results with norm values obtained in 1998 (1998 SF-36 US population norms), all scales were therefore not only expressed as absolute values but also norm based scored, i.e. each scale was scored to have the same average and the same standard deviation. We employed the standard (US) scoring algorithms, as has been recommended by Ware *et al*[19]. Results for the eight scales can then easily be compared directly since they are all standardized in relation to population norms.

Classification of malnutrition

The Subjective Global Assessment (SGA) was carried out using the protocol developed by Detsky *et al*[20]. It relies on the patient's history regarding weight loss, dietary intake, gastrointestinal symptoms, functional capacity, and physical signs of malnutrition (loss of subcutaneous fat or muscle mass, edema, ascites). Patients were classified as well nourished (A), moderately or suspected of being malnourished (B) or severely malnourished (C). Since subgroups of patients classified as SGA C were too small, comparative analyses were performed between well-nourished patients classified as SGA A and malnourished patients classified as either SGA B or C.

Anthropometry

Body weight was measured on light clothes with a portable electronic scale (seca 910, Hamburg Germany) to the nearest 0.1 kg and height was measured with a portable stadiometer (seca 220 telescopic measuring rod) to the nearest 0.1 cm. Weight and height were used to calculate BMI (weight (kg)/height (m)^2). Mid upper arm circumference (of the nondominant arm) was measured to the nearest 0.1 cm with a nonelastic tape measure and triceps skinfold was measured to the nearest 0.1 mm with a Holtain caliper (Crymych, UK) on the non dominant relaxed arm midway between the tip of the acromion and the olecranon process. Arm muscle area (AMA) and arm fat area (AFA) were calculated applying the formula by Gurney[21].

Body composition

BIA was performed using a BIA 2000M (Data Input GmbH, Darmstadt, Germany) applying alternating electric currents of 800 μA at 50 kHz. Patients were measured in the morning after an overnight fast, in the supine position with arms and legs abducted from the body. Source and sensor electrodes were placed on the dorsum of both hand and foot of the dominant side of the body. Total body water and fat free mass (FFM) were then calculated according to the formula by Kushner and Schoeller, body cell mass (BCM) and extracellular mass (ECM) were calculated as described previously[22]. BCM was also expressed corrected for height (BCM/m^2). ECM/BCM ratio was calculated to detect shifts between BCM and ECM.

Phase angle is a bioimpedance parameter that reflects the contribution between resistance, the pure opposition of a biological conductor to alternating electric current, and capacitance, which is the resisting effect produced by the tissue interfaces (arc tangent of this ratio transformed to degrees). It has been shown to be of clinical relevance in a number of disease settings[23,24].

Muscle function

Hand grip strength was measured both in the nondominant and in the dominant hand with a Digimax electronic dynamometer (Mechatronic GmbH, Germany). The patients performed the test while sitting comfortably with shoulder adducted and neutrally rotated, the elbow supported on a table and flexed to 90 degrees, forearm and wrist in neutral position. The patients were instructed to perform a maximal isometric contraction.

Table 2 Study population and differences between well and malnourished patients

Characteristics	Overall study population (n = 200)	Well nourished (SGA A) (n = 104)	Malnourished (SGA B C) (n = 96)	P
Gender (M/F)	94/106	44/60	50/46	NS
Age (yr)	54.9 ± 17.1	56.7 ± 16.7	52.9 ± 17.3	NS
Albumin (g/L)	37 ± 7.1	39 ± 7.0	35 ± 7.0	0.001
Body mass index (kg/m²)	23.7 ± 4.9	25.7 ± 4.6	21.6 ± 4.2	<0.000
Phase angle (°)	4.9 ± 1.1	5.2 ± 1.0	4.5 ± 1.1	<0.000
BCM/height (kg/m²)	8.1 ± 1.8	8.6 ± 1.7	7.4 ± 1.8	<0.000
ECM/BCM	1.3 ± 0.6	1.2 ± .4	1.4 ± .7	<0.000
Arm muscle area (mm²)	4327.5 ± 1261.8	4713.2 ± 1200.3	3916.1 ± 1200.1	<0.000
Hand grip strength (kg)	29.1 ± 11.0	30.6 ± 10.5	27.3 ± 11.4	0.030
Peak flow (L/min)	346.0 ± 118.4	353.4 ± 113.2	337.6 ± 124.2	NS

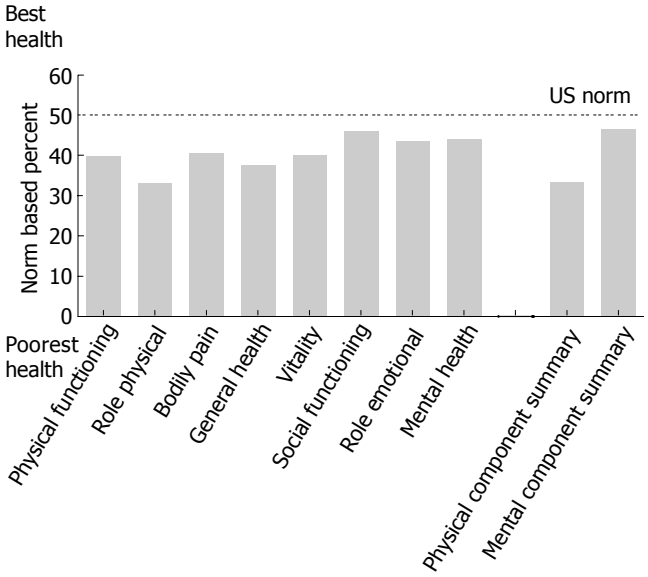


Figure 1 Quality of life in the study population.

Table 3 Study population and differences between liver cirrhosis and IBD patients

Differences	Liver cirrhosis	Inflammatory bowel disease	P
SGA A *vs* BC	34/27	33/36	NS
Gender (M/F)	33/28	29/40	NS
Age (yr)	56.8 ± 12.2	38.5 ± 13.4	< 0.000
Albumin (g/Ll)	33.0 ± 6.0	38.0 ± 7.0	< 0.000
Body mass index (kg/m²)	26.1 ± 4.9	21.2 ± 3.6	< 0.000
Phase angle (°)	4.7 ± 1.0	5.4 ± 1.2	< 0.000
BCM/height (kg/m²)	8.4 ± 1.9	7.9 ± 1.8	NS
ECM/BCM	1.4 ± .5	1.2 ± .8	< 0.000
Arm muscle area (mm²)	4606.3 ± 1485.9	3921.9 ± 1069.8	0.009
Hand grip strength (kg)	30.9 ± 11.6	31.5 ± 11.4	NS
Peak flow (L/min)	328.2 ± 116.9	375.8 ± 112.5	0.048

The test was repeated within 30 s and the highest value was recorded.

Peak expiratory flow was investigated by the Vitalograph© Peak Flow Meter. Patients were instructed to exhale as fast and as forcefully as possible. The test was repeated within 30 seconds and the highest value was recorded.

Statistical analysis

Statistical analysis was carried out using the software package SPSS© (version 11, SPSS Inc. Chicago, IL, USA). All data are given as mean ± SD. The Mann–Whitney U Test was used for comparison between groups. An acceptable level of statistical significance was established a priori at $P < 0.05$.

RESULTS

Patients' characteristics are given in Table 2. Patients classified moderately or severely malnourished (n = 96) by the Subjective Global Assessment were not significantly older than well nourished patients were. As anticipated however, they exhibited lower blood albumin levels, a significantly reduced body cell mass corrected for height and a significantly increased ECM/BCM ratio when compared to the well nourished patients. Body mass index and arm muscle area were also reduced. Moreover, muscle function as assessed by hand grip strength was significantly lower in the malnourished patients (Table 2). Peak flow was not different between well and malnourished patients.

All patients suffered impaired quality of life in all scales when compared with norm values from the US adult population (Figure 1). Characteristics of the liver cirrhosis and IBD patients are given in Table 3. IBD patients were significantly younger than liver cirrhosis patients and had higher serum albumin level. BCM did not differ between the two groups. IBD patients exhibited lower body

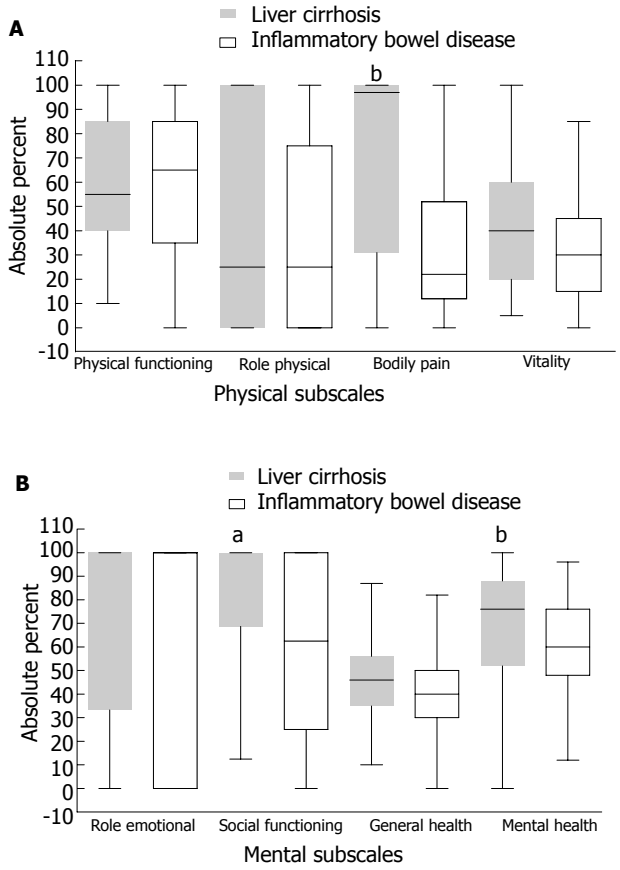


Figure 2 Quality of life ($^{a}P < 0.05$, $^{b}P < 0.01$).

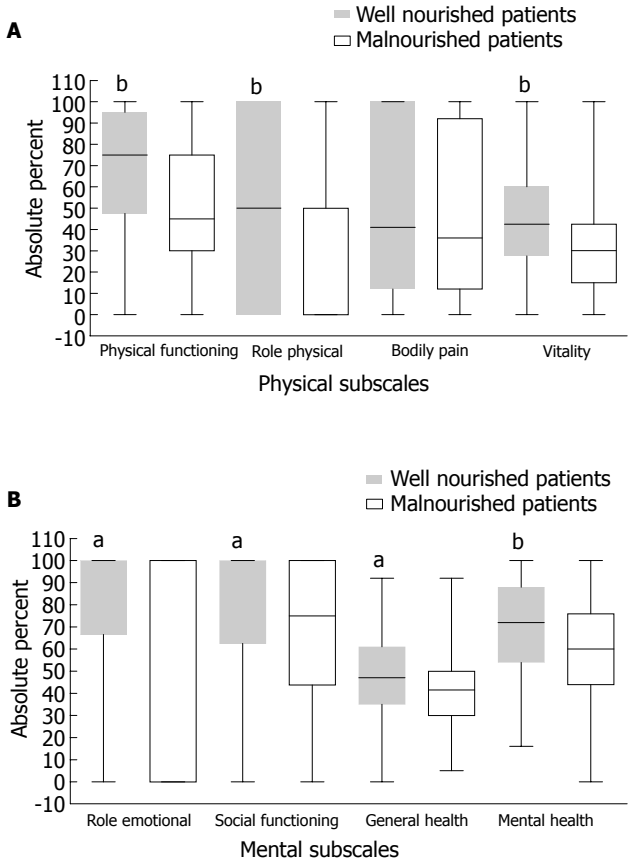


Figure 3 Quality of life in malnourished *vs* well nourished patients ($^{a}P < 0.05$, $^{b}P < 0.01$).

mass index, higher phase angle but reduced arm muscle area. ECM/BCM ratio was significantly also lower in IBD patients than liver cirrhosis patients. Hand grip strength was not different between the two groups but peak flow was higher in IBD patients. When evaluating their quality of life, IBD patients exhibited slightly lower quality of life values than liver cirrhosis patients did. This was significant in the scales concerning bodily pain ($P < 0.00001$), general health ($P < 0.00001$), mental health ($P = 0.031$) and social functioning ($P = 0.016$) (Figure 2).

Impact of malnutrition on parameters of quality of life

Malnourished patients from the overall study group suffered significantly impaired quality of life in both the mental and physical dimension of the quality of life. All subscales except bodily pain were reduced (Figure 3).

Evaluating the two chronic disease groups, malnourished liver cirrhosis patients experienced significantly lower quality of life in six out of the eight scales compared to well nourished liver cirrhosis patients. Malnourished IBD patients showed reduced quality of life in four out of the eight scales (Table 4).

DISCUSSION

In this study we investigated the quality of life of 200 patients with benign acute and chronic gastrointestinal disease in relation to their nutritional status. Quality of life in relation to nutritional status has been investigated in patients on haemodialysis, in institutionalized elderly and in patients with cancer[3-9,25,26]. At present however, there is not sufficient information on quality of life in relation to nutritional status in chronic benign gastroenterological disease, although malnutrition is frequently observed in these patients[27,28] and has well known adverse effects on clinical outcome[29-31]. In order to use quality of life as a clinical outcome parameter in these patients, it is therefore essential to enhance the understanding of quality of life and its association with nutritional status. We employed the SF 36 questionnaire which has been validated in various settings[32-34] and has the advantage of being disease unspecific. We moreover compared the results with values gathered in the 1998 US National Survey of Functional Status. Quality of life was generally impaired in our study population when compared to the norm values. Similar to the findings in other disease settings we also found that quality of life appeared to be compromised in malnutrition. Patients from the overall study group classified as malnourished experienced a significantly lower quality of life than the well nourished patients in all scales except in the perception of pain, leading to impairment of both the mental and the physical dimension of quality of life.

Among our study patients, the largest subgroups were patients with liver cirrhosis or IBD. Since health related quality of life obviously depends on underlying disease we also focused the evaluation on these patients.

Malnourished liver cirrhosis patients suffered statistically significant reductions in all scales except the

Table 4 Quality of life in malnourished *vs* wellnourished patients with either liver cirrhosis or inflammatory bowel disease (absolute percentage)

QOL scales	Liver cirrhosis			Inflammatory bowel disease		
	Wellnourished	Malnourished	*P*	Wellnourished	Malnourished	*P*
Physical functioning	74.0 ± 23.8	44.6 ± 22.1	0.000	66.6 ± 31.6	52.1 ± 28.4	0.036
Role physical	52.3 ± 42.6	22.2 ± 36.9	0.003	49.2 ± 45.1	24.4 ± 34.6	0.024
Bodily pain	88.9 ± 28.5	59.3 ± 47.4	0.007	67.7 ± 41.9	59.1 ± 46.5	NS
General health	82.6 ± 27.4	81.0 ± 28.7	NS	71.9 ± 31.6	51.1 ± 33.1	0.012
Vitality	73.2 ± 38.2	59.0 ± 39.4	NS	35.4 ± 33.5	37.7 ± 33.7	NS
Social functioning	51.8 ± 19.8	41.3 ± 16.1	0.041	42.1 ± 20.2	37.6 ± 13.5	NS
Role emotional	46.8 ± 21.3	31.7 ± 20.0	0.007	39.1 ± 23.7	26.9 ± 18.8	0.027
Mental health	78.1 ± 17.6	56.9 ± 25.9	0.001	64.9 ± 18.9	56.1 ± 21.8	NS

perception of social functioning and bodily pain whereas malnourished IBD patients were compromised in social functioning but mainly in the physical scales such as physical functioning, role physical and vitality.

One difficulty in evaluating our study results was to reliably differentiate between the effects of disease and the effects of malnutrition on quality of life, since they obviously are closely interrelated. Severity of disease is likely to trigger a worsening of nutritional status and malnutrition has well known adverse effects on the clinical outcome. Studies investigating health related quality of life in relation to disease severity do not show consistent findings. Kalantar-Zadeh *et al*[8] observed that quality of life assessed by the SF 36 had a strong association with disease severity judged by prospective hospitalization and mortality in patients on haemodialysis. Bianchi *et al*[10] showed a relationship between the disease severity index Child Pugh Score and the Psychological General Wellbeing Index. In patients with hepatocellular carcinoma however, quality of life was not primarily related to tumour mass or hepatocellular failure, but sleep disorders were shown to be strongly associated with quality of life. Similarly, in primary biliary cirrhosis, no associations between scales of the Nottingham health profile and biochemical liver tests, histological stages or duration of the disease were found[17]. Moreover, Hauser *et al* found psychiatric or active medical comorbidity to be of a larger influence than disease severity in a population with various liver diseases[35].

In this study, reliable data on disease severity were only available in liver cirrhosis and Crohn's Disease. In these groups, malnourished patients also experienced greater disease severity classified by the Child Pugh Score or the Crohn´s Disease Activity Index (data not shown). This clearly makes interpretation of the results more difficult and we consider it a limitation of the study. We cannot reliably conclude whether the observed impairments of quality of life in malnutrition are due exclusively to nutritional deprivation.

Malnutrition however is known to be associated with impaired functional status[1,2], worsened immune system[36], delayed recovery, higher mortality and morbidity[31]. The malnourished patients in our study population exhibited both lower muscle mass (as measured by body cell mass and arm muscle area) and muscle function and these changes were not attributable to age. It is therefore highly probable that malnutrition per se-defined mainly by weight loss in the preceding 6 months-has a measurable impact at least on the physical aspect of quality of life, which in the end is likely to affect the mental dimension as well.

Interestingly, overall quality of life was lower in the patients suffering from IBD than in liver cirrhosis patients, which was due to the perception of general health, mental health, vitality, social functioning and bodily pain. The physical scales physical functioning, role physical and vitality were not significantly higher in the IBD patients despite their younger age. There were no significant differences between patients with ulcerative colitis or Crohn's disease (data not shown). Considering that IBD in general is associated with a better prognosis and longer survival time than liver cirrhosis, these results are somewhat alarming. Our findings indicate that acute disease specific aspects such as diarrhoea and abdominal pain occurring in IBD rather than survival associated disease severity lead to a greater impairment of health-related quality of life. Marchesini *et al*[14] who studied quality of life in an Italian cohort of liver cirrhotics found similar associations between non life threatening symptoms such as muscle cramps and quality of life.

It is evident that subjective perception of quality of life depends on more than nutritional status or disease severity. Cancer patients currently on remission might e.g. experience a better quality of life than their healthy but overworked physician. When deciding on optimal therapy, it must also be taken into account that patients with impaired quality of life must be considered in need of intensified attention and care.

In conclusion, quality of life is impaired in benign gastrointestinal disease and becomes further compromised in malnutrition. It appears that liver cirrhosis patients experience a higher quality of life than IBD patients do, but the impact of malnutrition seems to be greater in liver cirrhosis than in IBD. Further studies are required to investi-

gate and identify therapy strategies that improve quality of life in chronic benign gastroenterological disease. Whether nutritional intervention is successful in enhancing quality of life in these patients remains to be studied.

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CLINICAL RESEARCH

Rectal nitric oxide as biomarker in the treatment of inflammatory bowel disease: Responders *versus* nonresponders

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Abstract

AIM: To explore rectal nitric oxide (NO) as biomarker of treatment response in ulcerative colitis (UC) and Crohn's disease (CD), and examine relationships between rectal NO, mucosal expression of NO synthases (NOS), and pro-inflammatory cytokines.

METHODS: Twenty-two patients with UC and 24 with CD were monitored during steroid treatment. Rectal NO levels were measured and clinical activities were assessed on days 1, 3, 7 and 28. Mucosal presence of NOS and pro-inflammatory cytokines were analyzed by immunohistochemistry and RT-PCR.

RESULTS: Active UC and CD displayed markedly increased rectal NO levels (10950 ± 7610 and 5 040 ± 1 280 parts per billion (ppb), respectively) as compared with the controls (154 ± 71 ppb, $P < 0.001$). Rectal NO correlated weakly with disease activity in both UC and CD ($r = 0.34$ for UC and $r = 0.48$ for CD, $P < 0.01$). In 12 patients, a steroid-refractory course led to colectomy. These patients had only slightly increased NO levels (UC: 620 ± 270 ppb; CD: 1260 ± 550 ppb) compared to those with a therapeutic response (UC: 18860 ± 530 ppb, $P < 0.001$; CD: 10060 ± 3200 ppb, $P < 0.05$).

CONCLUSION: Rectal NO level is a useful biomarker of treatment response in IBD as low NO levels predicts a poor clinical response to steroid treatment.

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Key words: Crohn's disease; Cytokines; Nitric oxide; Steroids; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory diseases sharing a clinical course with flares of disease, characterized by an increase of symptoms due to increased inflammatory activity of the intestinal mucosa. Symptom-based clinical activity indices are today's standard methods applied to monitor disease activity in clinical trials, but rarely used in clinical practice. Available indices have been criticized for depending almost exclusively on clinical features that are often subjective. The use of systemic markers of inflammation, such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet count and white blood cell (WBC) count are commonly used in clinical practice, but correlation to ongoing intestinal inflammation is poor[1,2]. An approach of direct assessment of mucosal inflammation has been promising, such as measurements of tumor necrosis factor (TNF)-α and interleukin (IL)-1β[3] and intestinal permeability tests[4]. The implementation of these tests is, however, restricted by a complicated analysis. The development of a more feasible objective marker of mucosal inflammation is, therefore, warranted. Data on local measurements of inflammatory products such as calprotectin, lactoferrin and nitric oxide (NO) in UC and CD have been promising[5].

We use a minimally invasive method employing chemiluminescence to measure rectal NO levels in conditions with inflamed intestinal mucosa[6,7]. Increased NO generation has been demonstrated in both UC and CD[6,8,9]. The excessive formation of NO is elaborated by inducible nitric oxide synthase (iNOS)[10,11]. Some groups have also reported increased endothelial NOS (eNOS) activity in inflamed mucosa in patients with UC[12] and iNOS expression restricted to epithelial cells[13,14]. Other groups have demonstrated iNOS expression also in

macrophages and granulocytes of the lamina propria[10,11].

The activation of iNOS is dependent on the transcription factor nuclear factor (NF)-κB, which is activated by bacterial products (e.g., endotoxins) and pro-inflammatory cytokines, such as TNF-α, IL-1β and interferon (INF)-γ. These pro-inflammatory cytokines act in synergy to stimulate NO production[15]. The aim of the present study was to investigate rectal NO levels as a biomarker of response in UC and CD during glucocorticosteroid (GCS) treatment, using established clinical indices as gold standard for clinical activity. We also assessed mucosal immunoreactivity to the cytokines TNF-α, IL-1β and INF-γ as well as activation of eNOS, iNOS and neuronal NOS (nNOS).

MATERIALS AND METHODS

Patients

Patients treated with prednisolone (0.5-1 mg/kg orally) at the Karolinska University Hospital for active UC or CD were eligible to enter this study. Forty-six consecutive patients, 22 with UC and 24 with CD, diagnosed by conventional endoscopic, radiological and histological criteria of IBD[16] were recruited to this study. Diagnosis of UC or CD was confirmed for all included patients at follow-up one-year after completion of the study. The majority of patients had concomitant medication with aminosalicylates, whereas no patient was on immunomodulators at baseline (Table 1).

The patients were studied at four different occasions during the first month of treatment, before onset of prednisolone treatment (d 1), and at follow-up during ongoing treatment on days 3, 7 and 28. A flexible sigmoidoscopy was performed at baseline, and at all follow-up visits. Disease activity was assessed using the Disease Activity Index (DAI)[17] for the patients with UC. The Harvey-Bradshaw Index (HBI)[18] was used for the patients with CD. The endoscopic classification was done according to the DAI score also for the CD patients.

At the last visit (d 28), the patients were divided into responders (remission) and non-responders (no remission) for further subgroup analysis. Remission was defined as DAI $\leqslant$ 2 in UC, and HBI $\leqslant$ 4 in CD, whereas non-responders were defined as DAI $\geqslant$ 3 in UC and HBI $\geqslant$ 5 in CD, respectively.

Two different control groups were used: the control for immunohistochemistry analyses consisted of six individuals undergone control colonoscopy after prior polypectomy, and the control for rectal NO consisted of 25 healthy volunteers with no history of gastrointestinal symptoms or abdominal surgery.

The study was approved by the Karolinska Institutet Ethics Committee. Written informed consent was taken from all patients.

Determination of rectal NO levels

NO was measured with a chemiluminescence analyzer (CLD 700, *Eco* Physics, Dürnten, Switzerland). The detection limit for NO was 1 part per billion (ppb). The analyzer was calibrated at known concentrations (100-10000 ppb) of NO in nitrogen gas (AGA, Lidingö, Sweden), administered through an electromagnetic flow controller (Environics, Middletown, CT, USA). The chemiluminescence assay is highly specific for NO without interference from other nitrogen oxides[19]. For sampling of rectal gas, we applied an all-silicon catheter (Argyle®, Sherwood Medical, Tullamore, Ireland) inserted into the rectum, using lubrication gel, free of local anesthetics, to a level 10 cm above the anal sphincter[7]. The balloon of the catheter was then inflated with 10 mL of ambient air containing less than 5 ppb of NO, and left for 10 min to equilibrate with gases in the rectum. Thereafter, the gas was withdrawn from the catheter balloon and diluted to a final volume of 50 mL before chemiluminescence analysis with correction for dilution. Analyses were performed within 15 min of sampling[7]. In cases where measurements exceeded the upper detection limit, further dilution steps were made in order to measure NO within the calibrated range.

Table 1 Patient characteristics

	Ulcerative colitis (n = 22)	Crohn's disease (n = 24)
Sex (M/F)	13/9	14/10
Age (yr) (mean and range)	41 (18 – 78)	42 (20 – 69)
Duration (yr) (mean and range)	7.4 (0 – 30)	9 (0 – 28)
Smoking habits (n)		
Active	2	3
Former	6	8
Non-smoker	7	9
Unknown	7	4
Concomitant medication (n)		
Aminosalicylates	18	12
Local steroids	6	2
Site of disease		
Ileocolonic		24
Rectum	3	
Proctosigmoiditis	6	
Extensive/total	13	
Disease activity index (mean and range)	9.4 (5 – 12)	
Harvey-Bradshaw index (mean and range)		14.9 (4 – 29)
Endoscopic score (mean and range)	2.2 (1-3)	2.1 (0-3)

Immunohistochemistry

Mucosal biopsies were sampled at sigmoidoscopy. Biopsy specimens were always taken in the vicinity of lesions or ulcerations. In case of colectomy (n =12), the surgical specimens were additionally used for analyses. Biopsies were kept in Histocon (Histolab, Gothenburg, Sweden) on ice and snap-frozen in liquid nitrogen within 30 min. Approximately 6 μm thick cryostat sections were mounted on gelatin-coated glass slides, and stored at -80 ℃. Before staining, the slides were thawed at room temperature and subsequently fixed in cold 20 g/L formaldehyde in phosphate-buffered saline (PBS). All following incubation and washing steps were performed in PBS supplemented with 1 g/L saponin (Sigma Chemicals, St Louis, MO, USA) to permeabilize cellular membranes[20]. Peroxidase activity was blocked with 3 g/L hydrogen peroxidase and 1 g/L sodium azide in PBS-saponin[21]. Human and goat serum

(Vector Laboratories Inc, Burlingame, CA, USA) and avidin/biotin blocking kit (Vector Laboratories) were used to block unspecific bindings. The sections were incubated overnight at 4℃ with mouse monoclonal antibodies to iNOS (NOS-IN 20 mg/L, Sigma), nNOS (NOS-BI 73 mg/L, Sigma), eNOS (NOS-EI 17 mg/L, Sigma), IL-1β (2D8 1.4 mg/L, ImmunoKontact, Abingdon, Oxon, UK), TNF-α (Mab I 10 mg/L + Mab II 14 mg/L, Pharmigen, San Diego, CA, USA), and to INF-γ (7-B6- 10 mg/L + 1-DIK 10 mg/L, Mabtech AB, Nacka, Sweden). Mouse IgG_1 (28 mg/L, Dako, Glostrup, Denmark) served as an isotype-matched negative control. Biotinylated goat anti-mouse IgG_1 (4 mg/L, Caltag laboratories, Burlingame, CA, USA) was used as secondary antibody, except for the eNOS staining (an IgA antibody) where it was substituted with biotinylated goat anti-mouse immunoglobulin (11 mg/L, Dako). The biotinylated secondary antibodies were followed by horse-radish peroxidase-conjugated avidin/biotin-complex (Vectastain, ABC-elite, Vector Laboratories). Positive peroxidase staining was developed with 3, 3´-diaminobenzidine (DAB peroxidase substrate kit, Vector Laboratories) and counterstained with haematoxylin (Histolab).

Quantification of immunohistochemical staining

All microscopic evaluations were performed by one investigator (T.L.) who was blinded to the clinical data as well as analyzed parameters (eNOS, nNOS, iNOS, TNF-α, IL-1β, INF-γ and IgG_1). To validate the observer's quantification, eight randomly chosen sections were additionally analyzed by a second observer (C. J.). Absolute correlation was seen between the two observers. Sections were analyzed using light microscope (Nikon Ltd, Tokyo, Japan). For each section, three different grid areas rich in positive cells of satisfactory technical quality were chosen for quantitative analysis of NOS and cytokine immunohistochemistry. Due to some background staining of the epithelial cells, we restricted our quantitative analysis to the lamina propria. For each area, the number of positive cells was counted at high-power magnification (× 400), thereafter the total number of positive cells was divided with the total grid area. For each section, the result was expressed as the mean number of positive cells per one grid area. For comparison, the percentage of positive cells in the lamina propria was also calculated. A high correlation between immunohistochemistry quantification expressed as positive cells per grid area and percentage positive cells of total cell number in lamina propria was observed ($r = 0.98$, $P < 0.001$).

Measurement of iNOS gene expression

Colonic biopsies were taken from four patients with CD and two with UC. The biopsies were collected from both normal and inflamed parts of the colon in each patient. Tissue samples were immediately placed in RNAlater (Qiagen, Hilden, Germany), stored for 24 h at 2-8℃ and then at -80℃.

After thawing, total RNA was isolated from the biopsies using the RNeasy mini kit (Qiagen). cDNA was synthesized using oligo $(dT)_{20}$ primers and SuperScript III enzyme (Invitrogen, Carlsbad, CA, USA). Semi-quantitative RT-PCR was performed using cDNA normalized against the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products obtained with primers specific for the human iNOS gene (sense 5´-CCAAAAGGGTCATCATCTCT-3´; antisense 5´-CCTGCTTCACCACCTTCTTG-3´) (GenBank accession number AF049656) in the linear amplification range (39 to 45 cycles) were electrophoresed on a 15 g/L agarose gel, photographed and bands quantified (Kodak Gel Logic 100 Imaging System, Rochester, NY, USA).

Table 2 Disease activity index, UC patients

t/d	All (n = 22)	Responder (n = 10)	Non-responder (n = 12)	Operated (n = 5)
1	9.4 (5-12)	9.5 (5-12)	9.4 (5-12)	11.2 (10-12)
3	7.4 (2-12)	7.1 (2-10)	7.6 (5-12)	12
7	5.7 (2-9)	4.8 (2-7)	7.2 (5-9)	NA
28	2.5 (0-8)	0.8 (0-2)	5 (3-8)	NA

Responder (DAI 2); non-responder (DAI ⩾ 3).

Table 3 Harvey-Bradshaw index, CD patients

t/d	All (n = 24)	Responder (n = 8)	Non responder (n = 16)	Operated (n = 7)
1	14.9 (4 - 29)	12.8 (4 - 26)	15.9 (9 - 29)	16.9 (11 - 29)
3	11.2 (2 - 22)	7.9 (2 - 13)	12.9 (2 - 22)	16.2 (11 - 22)
7	7.7 (1 - 19)	5.8 (1 - 19)	9.6 (2 - 13)	11.5 (10 - 13)
28	4.2 (1 - 9)	1.9 (1 - 3)	6.9 (4 - 9)	NA

Responder (HBI ⩽ 4); non-responder (HBI ⩾ 5).

Statistical analysis

For statistical analysis and graph plotting, GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used. Data were expressed as mean ± SE, and range where appropriate. Groups of independent data were compared using the Mann-Whitney U test. Intra-group variation was analyzed with the paired Wilcoxon test. Correlation coefficients between different analyses were calculated using the Spearman rank-test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical activity

Baseline DAI or HBI scores did not differ significantly between steroid responding *versus* non-responding patients and did not predict clinical outcome. On d 28, 45% (10/22) patients with UC and 33% (8/24) patients with CD were in remission (DAI ⩽ 2 and HBI ⩽ 4, respectively) (Table 2, Table 3). In 26.08% (12/46) patients (5 UC, 7 CD), colectomy was carried out on d 2-26 due to steroid-refractory severe disease. The DAI and HBI scores in this subgroup did not differ significantly on d 1 between subsequently operated patients and those responding to treatment (DAI: 9.5 ± 0.7 *vs* 11.2 ± 0.5 for responding and operated UC patients; and HBI: 12.8 ± 2.3 *vs* 16.9 ± 2.3

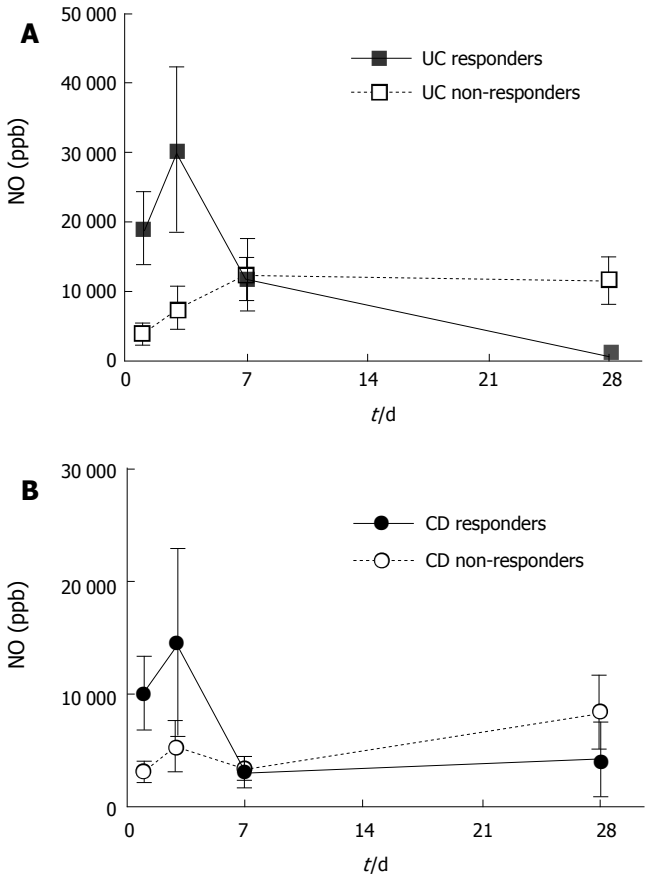


Figure 1 **A**: Rectal NO levels in UC; **B**: Rectal NO levels in CD.

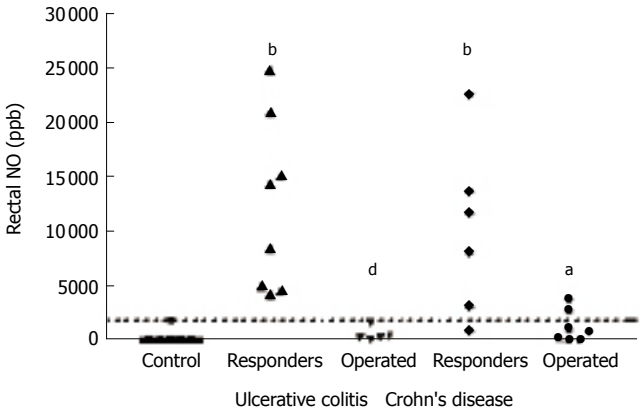


Figure 2 Rectal NO levels in the treatment of UC and CD patients on d 1. $^{b}P < 0.001$ *vs* Control; $^{d}P < 0.01$, $^{a}P < 0.05$ *vs* patients responding to treatment.

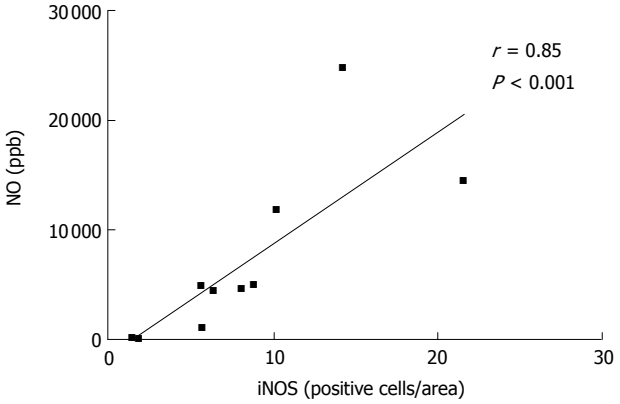


Figure 3 Correlation between mucosal iNOS-expressing cells and rectal NO levels in patients with UC.

for responding and operated CD patients) (Table 2, Table 3).

Rectal NO levels

On d 1, patients with active UC and CD had greatly increased rectal NO levels (10 950 ± 7610 and 5040 ± 1280 ppb, respectively) as compared with the controls (154 ± 71 ppb, all $P < 0.001$). Repeat measurements of rectal NO showed a numerical increase on d 3 compared to d 1, after which a decrease was seen (Figure 1). This pattern of NO release was mainly attributed to the patients responding to steroid treatment, whereas non-responders displayed a less prominent increase on day 3, and showed no subsequent decrease (Figure 1). For steroid-responding patients, rectal NO levels decreased significantly between d 1 and 28 (from 18 860 ± 5390 to 850 ± 450 ppb in UC, and 10 060 ± 3200 to 4130 ± 3380 ppb in CD, $P < 0.001$ and $P < 0.05$, respectively).

Rectal NO levels were correlated weakly with clinical activity scores of the whole study population, DAI for UC ($r = 0.34$, $P < 0.01$), and HBI for CD ($r = 0.48$, $P < 0.01$). However, the association was clear-cut in the group of responders to treatment ($r = 0.72$ for UC and $r = 0.64$ for CD, all $P < 0.001$).

A different rectal NO pattern was seen in the subgroup of patients in whom colectomy was carried out. The rectal NO levels of these patients were significantly lower at baseline (620 ± 270 ppb in UC, and 1260 ± 550 ppb in CD) than corresponding values in the patients with a treatment response ($P < 0.001$ for UC and $P < 0.05$ for CD) (Figure 2). Applying a cut-off level of rectal NO at 2000 ppb in combination with DAI ⩾ 10 for patients with UC identified all five patients with a steroid-refractory disease, leading to colectomy within 7 d, Whereas none of the UC patients responding to treatment combined rectal NO ⩽ 2000 ppb and DAI ⩾ 10. For CD patients, the same cut-off of NO ⩽ 2000 ppb in combination with HBI ⩾ 10 detected 5 of 7 patients subsequently operated, and was seen in 3 CD patients not leading to surgery.

Expression of NO synthases and pro-inflammatory cytokines

The number of iNOS-expressing cells, as judged by immunohistochemistry, was significantly higher in patients with UC and CD on d 1 as compared with the healthy controls (all $P < 0.001$). Furthermore, the number of iNOS-positive cells decreased between d 1 and 28 (Figure 4), reaching a borderline significance by pooling UC and CD patients ($P = 0.064$). The number of iNOS-positive cells was obviously correlated with rectal NO levels in patients with UC ($r = 0.53$, $P < 0.01$). This association was strengthened by analyzing the patients responding to treatment ($r = 0.85$, $P < 0.01$) (Figure 3).

However, no correlation was found between number of iNOS-positive cells and rectal NO levels in patients with CD. The majority of iNOS-positive cells in lamina

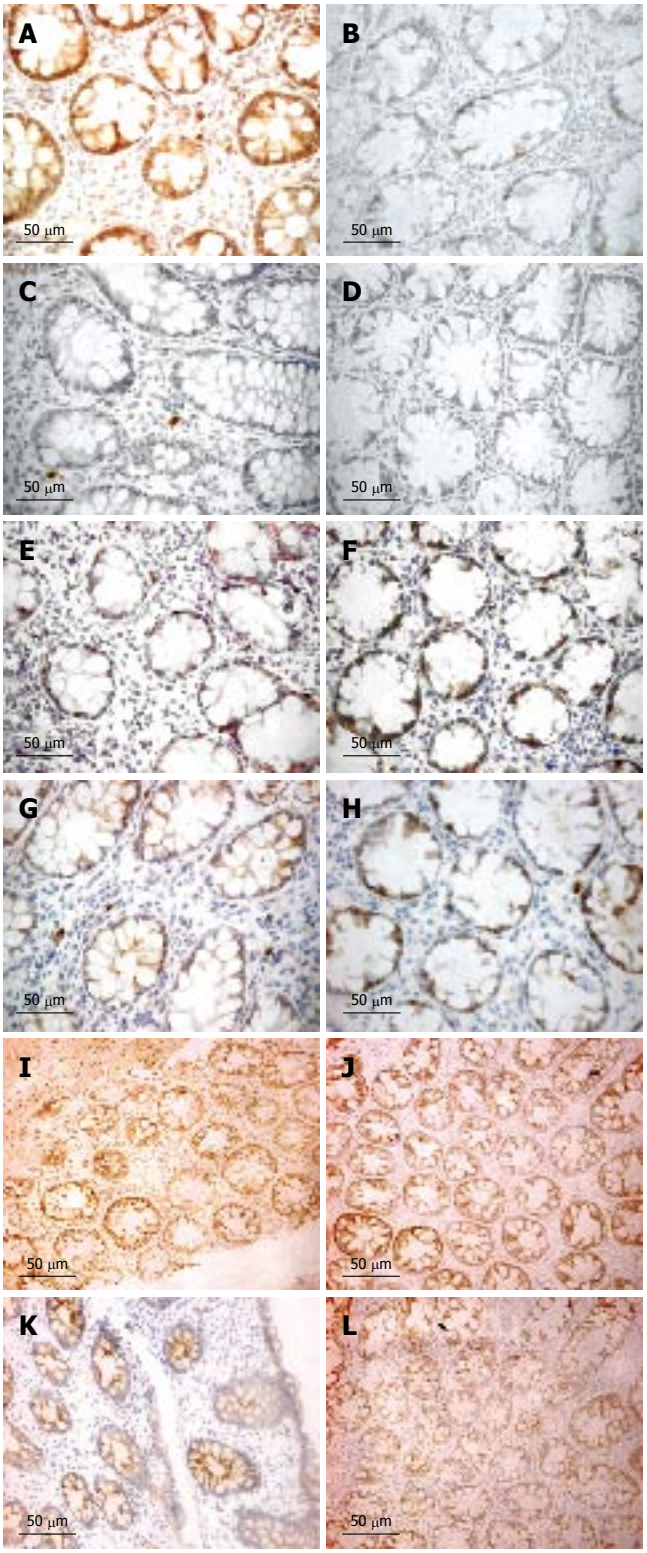


Figure 4 **A** Colonic histology of CD patients (**A**, **C**, **E**, **G**) before and (**B**, **D**, **F**, **G**) after treatment on d 28. Antibodies against iNOS (**A**, **B**), TNF-α (**C**, **D**), IL-1β (**E**, **F**) and INF-γ (**G**, **H**); Colonic histology of healthy control. Antibodies against iNOS (**I**), TNF-α (**J**), IL-1β (**K**) and INF-γ (**L**).

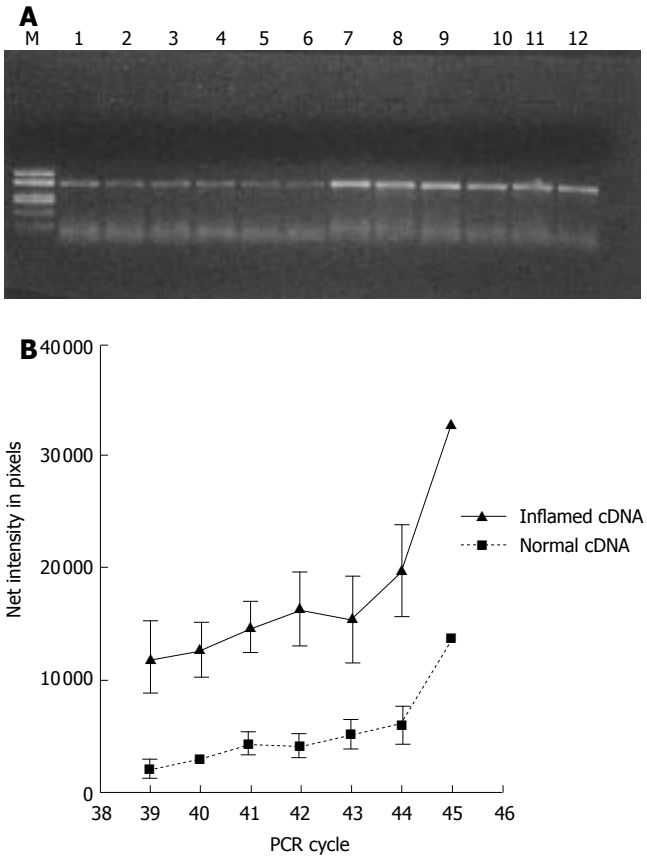


Figure 5 **A**: Semi-quantitative PCR iNOS gene of cDNA from non-inflamed (lanes 1-6), and inflamed (lanes 7-12) colon biopsies from a patient with ulcerative colitis; **B**: Difference in semi-quantitative PCR iNOS gene expression.

propria had the morphology of polymorphonuclear leukocytes (Figure 4).

Semi-quantitative RT-PCR showed over-expression of iNOS mRNA in the inflamed compared to normal parts of the mucosa ($P < 0.05$) (Figure 5). Presence of eNOS-positive cells was abundant in all sections and localized to the epithelium, but showed no temporal relationship with rectal NO levels. Recovery of nNOS staining was slight and, when occurring, mostly seen in the submucosa in nerve cells and randomly in mononuclear cells of the lamina propria.

TNF-α, IL-1β and INF-γ expressions, as judged by immunohistochemistry, were restricted to mononuclear cells in the lamina propria. IL-1β expression was significantly increased on d 1 compared to healthy controls ($P < 0.05$), whereas TNF-α and INF-γ were not. However, both TNF-α and IL-1β decreased numerically between d 1 and 28 for UC and CD (Figure 4). By pooling data from UC and CD patients, a significant reduction was seen ($P < 0.05$ for both TNF-α and IL-1β immunoreactivity). INF-γ did not change between d 1 and 28. A marked increase of iNOS-expressing cells was correlated with TNF-α expression in both UC ($r = 0.46$, $P < 0.05$) and CD ($r = 0.44$, $P < 0.05$). By pooling data for UC and CD, a weak correlation was detected for IL-1β ($r = 0.33$, $P < 0.05$). No correlation was seen between the numbers of iNOS and INF-γ-expressing cells.

DISCUSSION

This study provide evidence suggesting that the greatly increased rectal NO levels seen in active UC and CD are elaborated by high iNOS activity. In our study, a strong correlation was seen between rectal NO levels and clinical

activity indices in patients responding to steroid treatment. A relationship between ongoing inflammatory activity and increased NO production in the gut is well established[6,8]. In agreement with this, we found a correlation with clinical disease activity indices in both UC and CD.

Taking into account the relatively low number of patients recruited, the subgroup analysis have to be interpretated with caution. In the non-responding group, no correlation was seen between rectal NO and clinical activity indices. This is in part explained by the aberrant rectal NO pattern in the 12 patients who subsequently underwent colectomy due to severe disease. The finding that baseline rectal NO $\leqslant$ 2000 ppb in severe UC detected all patients in need of surgery within 7 d due to steroid-refractory disease makes it tempting to suggest rectal NO measurement as a predictive marker of the need of colectomy in active UC. In CD patients, rectal NO seems to be a less strong marker for steroid-response, which is in agreement with previous studies demonstrating a 70% endoscopic remission post-treatment in UC as compared with only 13% endoscopic remission in CD[22,23], as well as a recent study by Costa *et al*[24] showing that fecal calprotectin is a stronger marker for relapse in UC than in CD[24].

Due to the high affinity of NO to hemoglobin[25], one may argue that luminal NO is scavenged by blood in the colon, which should cause falsely low rectal NO levels in the most severe UC and CD cases. This is however unlikely since other severe cases in our group displayed high rectal NO levels in the presence of overt rectal bleeding.

The role of NO in intestinal inflammation is unclear, as both pro-inflammatory and tissue-protective properties have been demonstrated[26]. Our observation that high rectal NO levels at the first visit was associated with a favorable clinical outcome is consistent with the idea that NO may act as an endogenous inhibitor of an aggregated immune response. Tissue-protective properties of NO have been shown in animal models of colitis[27]. The finding that NO production in collagenous colitis, a chronic inflammatory bowel disease without mucosal cell damage[28], might be even greater than in active IBD supports the concept of NO acting as a modulator of inflammatory activity in the gut. An uncontrolled chronic inflammation could, however, lead to an intracellular depletion of L-arginine, during which NOS will produce O_2^- instead of NO, O_2^- will then immediately react with NO to form $ONOO^-$, considered to be highly cytotoxic[29].

Genetic polymorphisms of iNOS might be a plausible explanation for the different rectal NO patterns seen in the group of responding *versus* non-responding patients. iNOS polymorphism has previously been associated with outcome variables in different diagnoses[30-32].

There is substantial evidence pointing towards iNOS-expressing epithelial cells lining the mucosa as a major site of NO production in intestinal inflammation[13-15]. We could not confirm this finding in our present study as we encountered unspecific staining of the epithelial cells, but we consider the correlation between the number of iNOS-positive cells in lamina propria and the production of NO, measured as rectal NO levels, as circumstantial evidence, suggesting the importance of these cells in the production of the increased NO levels seen in active UC and CD. This is supported not only by earlier studies showing iNOS-positive macrophages and granulocytes in the mucosa in IBD[11], but also by our present finding of a molecular activation of iNOS within the tissue. Our morphological examination of iNOS-positive cells ascribes polymorphonuclear leukocytes as a plausible cellular source of NO production.

Whether the constitutive forms of NOS contribute to the increased NO production in IBD is unclear. For eNOS or nNOS, we could not detect any differences in staining between healthy controls, active IBD or IBD in remission. Little information is available on possible changes in eNOS and nNOS in IBD in literature. One previous study reports findings in line with our results[14], i.e. activation of mainly iNOS, whereas another group claims specific changes in eNOS and nNOS expression in UC[12].

Determination of tissue cytokine levels in IBD has resulted in disparate results. In general, pro-inflammatory cytokines (TNF-α, IL-1β and INF-γ) are believed to have a regulatory function in the activated immune response in IBD. Clearly the last years' development has pin-pointed the key role of TNF-α in CD[3,21,33]. In line with some previous studies, we failed to show increased TNF-α expression in IBD compared to controls[34,35]. However, we found a decrease in TNF-α expression as a response to treatment, supporting data demonstrating an association between TNF-α expression and active IBD[3,36,37]. Our data showing increased IL-1β expression in active IBD compared to healthy controls are consistent with earlier studies providing evidence that IL-1β has a central role in the mucosal inflammation as seen in IBD[34,36,37]. In our study, we found no support for INF-γ as a marker of disease activity in IBD.

We found a correlation between TNF-α and IL-1β expression and iNOS expression, supporting the concept that these cytokines may have a role in inducing NO production[15].

In summary, our study shows that active UC and CD are associated with highly increased rectal NO levels. Rectal NO levels decreases in response to steroid treatment, hence offering a plausible and feasible objective method to monitor disease activity in IBD. Low rectal NO levels $\leqslant$ 2000 ppb might be a predictive marker for steroid-refractory IBD requiring acute colectomy. Furthermore, our data suggest that the major part of the increased NO production seen in active IBD is elaborated by the inducible form of NOS.

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CLINICAL RESEARCH

Effects of adacolumn selective leukocytapheresis on plasma cytokines during active disease in patients with active ulcerative colitis

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Abstract

AIM: To investigate the relationship between ulcerative colitis (UC) clinical activity index (CAI) and circulating levels of IL-1ra, IL-10, IL-6 and IL-18.

METHODS: Blood levels of IL-1ra, IL-10, IL-6 and IL-18 were measured in 31 patients with active UC, the mean CAI was 11.1, ranging from 5-25; and 12 healthy individuals as controls. Patients were given granulocyte and monocyte adsorptive apheresis (GMA) with Adacolumn. Leucocytes which bear the FcγR and complement receptors were adsorbed to the column leucocytapheresis carriers. Each patient could receive up to 11 GMA sessions over 8 wk.

RESULTS: We found strong correlations between CAI and IL-10 (r = 0.827, P < 0.001), IL-6 (r = 0.785, P < 0.001) and IL-18 (r = 0.791, P < 0.001). IL-1ra was not correlated with CAI. Following GMA therapy, 24 of the 31 patients achieved remission and the levels of all 4 cytokines fell to the levels in healthy controls. Further, blood levels of IL-1ra and IL-10 increased at the column outflow and inflow at 60 min suggesting release from leucocytes that adhered to the carriers.

CONCLUSION: Elevated blood levels of IL-6 and IL-18 together with peripheral blood granulocytes and monocytes/macrophages in patients with active UC show activative behaviour and increased survival time can be pro-inflammatory and the targets of GMA therapy.

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Key words: Interleukin-1 receptor antagonist; Interleukin-6; Interleukin-10; Interleukin-18; Ulcerative colitis

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INTRODUCTION

Factors which initiate and perpetuate ulcerative colitis (UC) are not well understood. However, the condition is often associated with elevated circulating granulocytes and monocytes/macrophages[1-5] that show activative behaviour and increased survival time[6-10]. Another feature of active UC is the increased generation and activities of inflammatory cytokines which can initiate and perpetuate the disease[11-15]. Furthermore, recent clinical and basic research data indicate that the clinical relapse of UC is mediated by an unchecked influx of granulocytes and monocytes/macrophages into the mucosal tissue[16-19]. The level of neutrophils in the mucosa was quantitatively related to the severity of intestinal inflammation and relapse both in UC and Crohn's disease[16].

Granulocytes and monocytes/macrophages are major sources of inflammatory cytokines[20-24] and therefore, it is assumed that high and activated peripheral blood lev-

els of these leucocytes can be a major pro-inflammatory condition. Factors believed to contribute to the elevated neutrophil counts and increased survival time include inflammatory cytokines[8] and paradoxically corticosteroids[9] that are given to most patients with severe active UC. The circulating levels of anti- and pro-inflammatory cytokines are likely to follow leucocyte levels or activities and are potentially related to disease activity.

However, leucocytes produce proinflammatory as well as anti-inflammatory cytokines. Thus, interleukin-6 (IL-6) produced by neutrophils and monocytes/macrophages (as well as other cell types including endothelial cells) is reported to play a major role in neutrophil and monocyte recruitment during inflammation[15]. Similarly, IL-18 produced by monocytes/macrophages is thought to be a potent inducer of interferon-γ (IFN-γ) production, hence contributing to the induction of Th1 responses[23, 24] A similar scenario may be described for anti-inflammatory cytokines. Thus, elevated level of IL-1 receptor antagonist (IL-1ra) is considered to reflect a natural compensatory mechanism to counter the activities of the pro-inflammatory cytokine IL-1β in inflammatory diseases[25-26]. Similarly, during inflammation, production of IL-10 increases and potentially can switch off the production of pro-inflammatory cytokines including IFN-γ, IL-1β, IL-6, IL-8, IL-12 and tissue necrosis factor (TNF-α).

Therefore, there are cytokines which can initiate and perpetuate inflammation and others which mitigate the inflammation. Accordingly, cytokines currently represent the best validated therapeutic targets[25-30]. With this in mind, we studied the circulating levels of 2 major anti-inflammatory and 2 major pro-inflammatory cytokines during active UC and during remission. IL-10 and IL-1ra were chosen as two known anti-inflammatory cytokines while IL-6 and IL-18 were considered to represent two typical pro-inflammatory cytokines. Since peripheral blood granulocytes and monocytes/macrophages are thought to represent major sources of both pro- and anti-inflammatory cytokines[1-6], we targeted these cells using granulocyte and monocyte/macrophage adsorptive apheresis (GMA) with Adacolumn[4,5].

MATERIALS AND METHODS

Study objectives

First, to see the relationship between CAI and circulating levels of the two major anti-inflammatory cytokines IL-10, IL-1ra and two pro-inflammatory cytokines IL-6 and IL-18 during active UC. Second, to investigate the effects of adsorptive granulocytes and monocyte/macrophage apheresis (GMA) on the levels of these cytokines and CAI. This was to provide an insight into the mechanisms of clinical efficacy of GMA with Adacolumn presently and hitherto reported[4,5,31]. Third, to identify predictors of response to GMA.

Patients

The demography of the 31 patients included in this study is presented in the Table 1. There were 17 males and 14 females, mean age 34.3 years, range 14-58 years.

Table 1 Demography of 31 patients with active ulcerative colitis who were investigated in this study

Backgroung	Measurement	
Male/Female	17/14	
Age, yr (range)	34.5-15.3	(14-58)
Age at first attack (yr)	29.1-11.9	(12-54)
Duration of UC (yr)	5.9-7.2	(0.5-26)
Number of relapses	4.3-3.9	(1-15)
Classification of severity		
Mild		4
Moderate		16
Severe		11
Mean (range) clinical activity index (CAI)[33]		11.1(5-25)
Total colitis		24
Left sided colitis		7

CAI was determined according to Rachmilewitz[32]. UC was severe in 11, moderate in 16 and mild in 4. All patients had been treated with conventional medications including 5-aminosalicylic acid (5-ASA) and prednisolone prior to entry. Eighteen of the 31 patients were steroid dependent , 9 were steroid refractory and 4 were steroid naive. The conventional medication was continued and the steroid dose was tapered when CAI score decreased to remission level (4 or less). Twelve healthy individuals of the same age range served as a control group.

Granulocyte and monocyte adsorptive apheresis

GMA was performed with Adacolumn precisely as described previously[4,5,20,31,33]. In Japan, GMA with Adacolumn has been approved by the Ministry of Health for public funding to treat patients with active UC. Adacolumns were purchased from Japan Immunoresearch Laboratories (Takasaki, Japan). Each patient could receive up to 11 GMA sessions. Patients with CAI > 12 received 2 sessions/wk in the first 3 wk and then one session/wk. During wk 6, CAI scores were determined and GMA was continued until patients had received up to 11 sessions. This therapy was added to the patients' ongoing treatment following a relapse or worsening UC symptoms. No additional conventional medication was given during this study.

Assessment of response to therapy

CAI equal or greater than 5 was the entry criteria. Clinical remission was defined as a CAI decrease to 4 or less and mucosal vascular patterns had become visible (at least partly). When CAI had fallen, but was still above 4, the patient was considered to have improved.

Measurement of cytokines

Peripheral blood was sampled directly via a venepuncture in the antecubital vein and also from the Adacolumn inflow at the start of GMA and then at the end of the 60 minutes GMA session from the column inflow and outflow (blood which was returning to patients). IL-1ra, IL-10, IL-6 and IL-18 were measured using enzyme linked immuno-sorbent assays (ELISA). Human IL-1ra ELISA

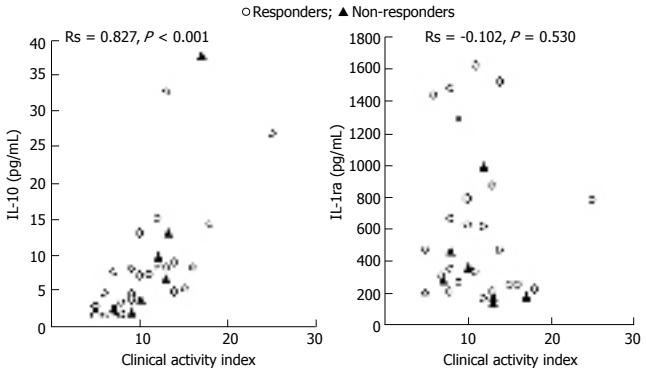


Figure 1 The relationship between blood levels of IL-10 and IL-1ra as measured in serum samples and the ulcerative colitis CAI in 31 patients with UC.

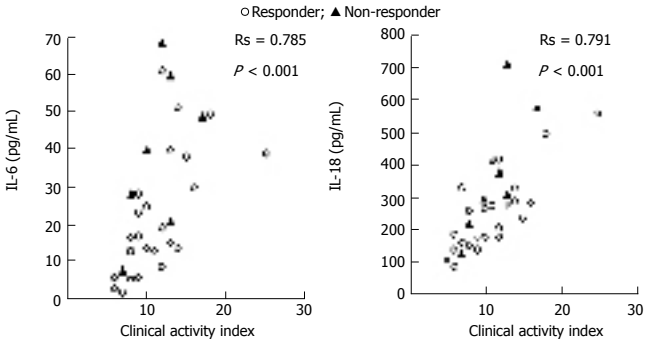


Figure 2 The relationship between blood levels of IL-6 and IL-18 and CAI in 31 patients with UC.

kit was from R&D systems (Minneapolis, USA); the ELISA kit for IL-10 was from Biosource Europe SA (Nivelles, Belgium); human IL-18 ELISA Kit was from MBL (Medical and Biological Laboratories, Nagoya Japan) and the ELISA kit for IL-6 was from FUJIREBIO Inc. (Tokyo). All assays were done at an special research laboratory (SRL) in blind manner and the data were processed by an individual who was unaware of the subjects' clinical conditions or the purpose of the study.

Ethics

As indicated above, Adacolumn is officially approved in Japan for the treatment of patients with active UC. However, when blood samples were required for research other than for routine clinical laboratory, patients were informed of the extra volume of blood to be taken and the purpose of the blood sample. All patients we consulted agreed to donate blood samples for assaying cytokines. They were advised that refusal to donate blood will not jeopardize their future treatment and care.

Statistical analysis

The amounts of IL-1ra, IL-10, IL-6 and IL-18 are presented as individual observations and comparisons are made using the Stat View Software. Statistical tests were the Mann-Whitney U test, Scheffe's test, the Turkey-Kramer test or the Spearman's rank correlation test, indicated in the figure legends. A significance level of 0.05 was used for all statistical tests, and two-tailed tests were applied when appropriate.

RESULTS

Blood levels of anti- and pro-inflammatory cytokines during active UC

The relationship between CAI and blood levels of IL-10 and IL-1ra in 31 patients is presented in Figure 1. The Spearman's rank correlation coefficient (Rs) showed a strong positive relationship between blood levels of IL-10 and CAI, while no correlation was found between blood levels of IL-1ra and CAI. Similarly, the majority of non-responders to GMA (albeit a few) had low blood levels of IL-1ra, while no obvious difference between responders and non-responders to GMA was seen for circulating IL-10 levels. Likewise, Figure 2 shows the relationship between blood levels of IL-6, IL-18 and CAI in the 31 patients. Also for these two pro-inflammatory cytokines, Rs shows a strong positive correlation with CAI. Most of the few non-responders to GMA had high blood levels of IL-6 and IL-18.

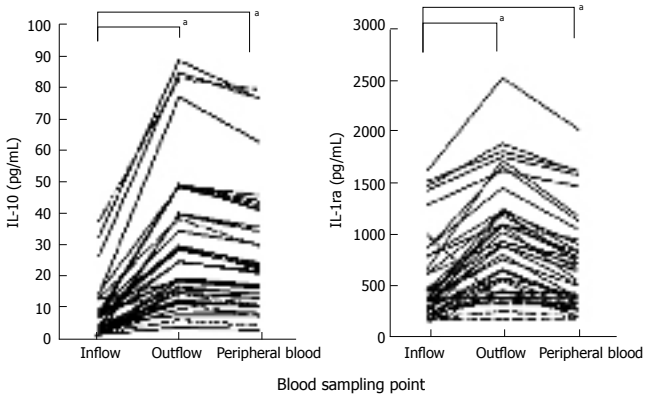


Figure 3 Blood levels of IL-10 and IL-1ra at the Adacolumn inflow (peripheral blood) at the start of GMA therapy and outflow at the end of the 60 min GMA and again peripheral blood just after the completion of 60 minutes GMA therapy in 31 patients with active UC. [a]$P < 0.05$ *vs* inflow by Turkey-Kramer test.

Blood levels of anti- and pro-inflammatory cytokines during GMA

Figure 3 shows the changes in blood levels of IL-10 and IL-1ra at the Adacolumn inflow (peripheral blood) at the start of GMA therapy and outflow at the end of the 60-min GMA and again peripheral blood just after the completion of GMA in the 31 patients. The dotted lines represent patients who did not respond to GMA during the observation period. This figure shows that blood levels of both IL-10 and IL-1ra are significantly higher in the column outflow relative to inflow and this accounts for the elevated levels of these cytokines in the peripheral blood during the GMA procedure. We found strong correlations between ulcerative clinical activity index (CAI) and circulating levels of IL-10, IL-6 and IL-18. In contrast, IL-1ra was high in most patients during active UC, but did not show correlation with CAI. However, the circulating levels of all 4 cytokines were low during remission, similar to the levels in healthy controls. It is inferred that the increments in IL-1ra and IL-10 in the column outflow are due to release from monocytes/macrophages and neutrophils

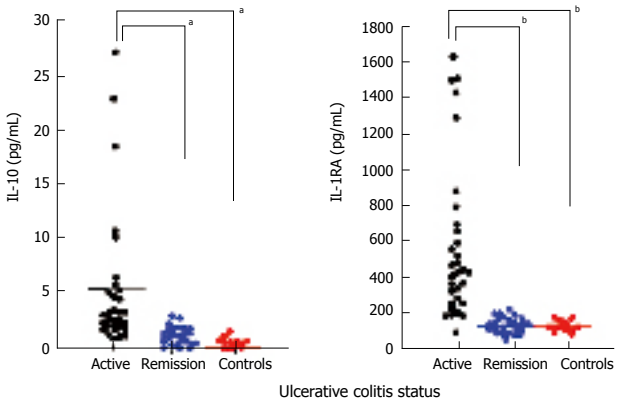


Figure 4 Blood levels of IL-10 and IL-1ra in patients with UC during active disease (n = 31), when in remission (n = 24) and in age matched controls (n = 12). [a]$P < 0.05$, [b]$P < 0.01$ *vs* active by Scheffe's test.

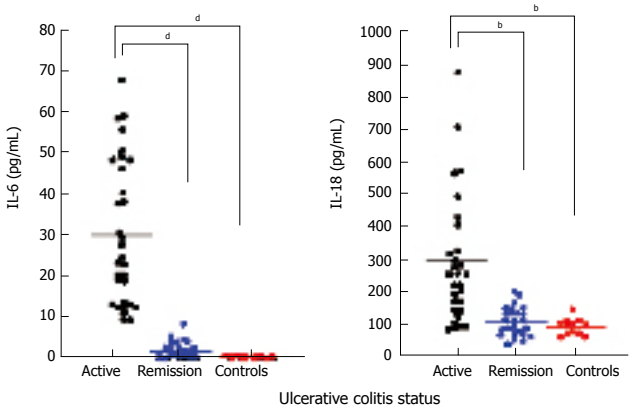


Figure 5 Blood levels of IL-6 and IL-18 (measured in serum samples) in patients with UC during active disease (n = 31), when in remission (n = 24) and in age matched controls (n = 12). [b]$P < 0.01$, [d]$P < 0.001$ *vs* active by Scheffe's test.

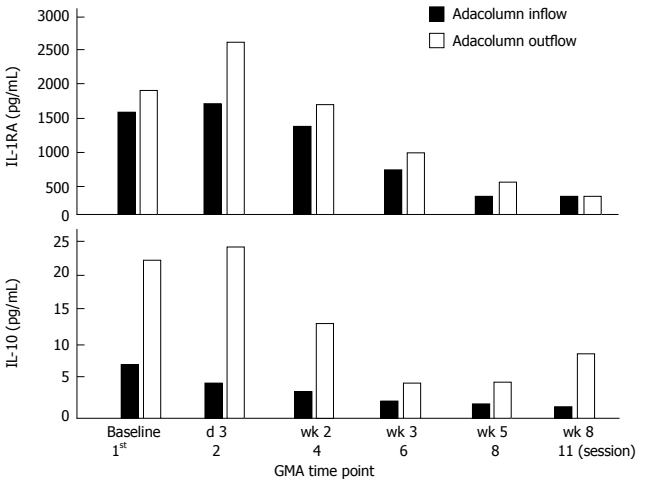


Figure 6 Changes in blood levels of IL-10 and IL-1ra in one typical case during an 8 wk GMA course (up to 11 GMA sessions).

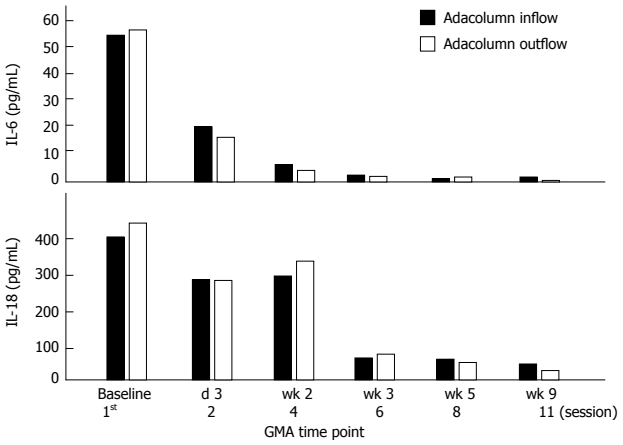


Figure 7 Similar to the data shown in Figure 6, here we see changes in blood levels of IL-6 and IL-18 were seen in one typical case during an 8 wk GMA course.

that adhere to the Adacolumn leucocytapheresis carriers, with additional contributions from activated Th2 and B lymphocytes (IL-10). Blood levels of IL-6 and IL-18 did not change significantly during the 60 min GMA procedure. The likely explanation could be inactivation within the column by a strong flux of proteases and active oxygen derivatives released by adherent leucocytes.

Blood levels of anti- and pro-inflammatory cytokines during active UC and remission

Figure 4 shows blood levels of IL-10 and IL-1ra in patients with UC during active disease (n = 31), when in remission (n = 12) and in age matched controls (n = 12). The results show wide variations in the blood levels of these two anti-inflammatory cytokines during active disease, but for both cytokines, the levels are very low during remission, similar to the levels in healthy controls. The 12 patients in remission were from the 31 patients with active disease who achieved remission following GMA (wk 12).

Blood levels of IL-6 and IL-18 are presented in Figure 5. The data for these two pro-inflammatory cytokines are mirror images of the data in Figure 4 for two anti-inflammatory cytokines. The results show wide variations in the blood levels of these two cytokines during active disease, but for both cytokines, the levels are very low during remission, similar to the levels in healthy controls.

Blood levels of anti- and pro-inflammatory cytokines during GMA course

Figure 6 shows the changes in blood levels of IL-10 and IL-1ra in one typical case during an 8 wk GMA course. Two prominent features can be seen: higher levels during active disease; in the column outflow and decline with the number of GMA sessions. This is a case who responded to GMA and achieved remission during this therapy.

Figure 7 shows IL-6 and IL-18 levels in one typical case during an 8 wk GMA course. Also for these two pro-inflammatory cytokines, the levels were high during active disease and fell rapidly (IL-6) during the course of GMA therapy. However, unlike IL-10 and IL-1ra, the levels of these two cytokines did not increase in the column outflow.

Changes in CAI associated with GMA

Patients received up to 11 GMA sessions to deplete the activated and excessive peripheral blood granulocytes and

monocytes/macrophages. There was a significant ($P < 0.05$) fall in CAI at wk 6 and after 11 sessions, 24 of the 31 patients were in clinical remission (CAI ⩽ 4). The mean CAI value at entry was 11.1, ranging 5-25. The corresponding values one week after the last treatment session were 2.4, ranging 0-15.

Safety of GMA

All patients completed their GMA therapy, compliance was excellent. GMA was safe, no severe side effects were seen during or after the GMA procedures. Transient flushing and mild headache were seen in a small number of patients, similar to the side effects reported in previous GMA studies[4,31,33].

DISCUSSION

Factors which initiate and perpetuate or exacerbate UC are yet to be characterized. The current view is that treatment strategies based on understanding of the factors that initiate or exacerbate UC (targeted therapy) should have greater efficacy margins and less treatment-related side effects[11,19,34,35]. However, UC is an inflammatory disorder reflecting unphysiological activities of pro-inflammatory cytokines and an over-exuberant intestinal inflammatory response[11,34,35]. Hence, it is assumed that cytokines can initiate and perpetuate inflammation and currently represent the best therapeutic targets. With this in mind, we studied their circulating levels during active disease and remission. IL-10 and IL-1ra were chosen as two typical anti-inflammatory cytokines while IL-6 and IL-18 were considered to represent two typical pro-inflammatory cytokines. The circulating levels of all 4 cytokines were high in most patients during active UC, but low during remission, similar to the levels in healthy controls. Further more, most non-responders to GMA had low blood levels of IL-1ra. This study did not measure mucosal tissue levels of these 4 cytokines which might be more appropriate to intestinal inflammation. Since monocytes/macrophages and granulocytes are major sources of both pro- and anti-inflammatory cytokines, this might suggest that these leucocytes contribute to the circulating levels of cytokines. Accordingly, we targeted these leucocytes using GMA with Adacolumn. The column carriers adsorb about 65% of granulocytes, 55% of monocytes and a small fraction of lymphocytes from the blood in the column (FcγR and complement receptors bearing leucocytes). The procedure is followed by a marked increase in peripheral blood lymphocyte counts, notably CD4+ T cells[5] and CD10 negative (immature) neutrophils[36].

The major cellular sources of IL-1ra are known to be monocytes/macrophages and polymorphonuclear granulocytes[37] while the major cellular sources of IL-10 are reported to be monocytes/macrophages, also Th2 cells and B lymphocytes[28]. Similarly, IL-6 is produced by neutrophils, monocytes/macrophages as well as endothelial cells[15] while IL-18 is produced by monocytes/macrophages, epithelial and Kupffer cells[23,24]. Each of these cytokines has a different profile of inflammatory or anti-inflammatory action. Both IL-1ra and IL-10 are reported to have strong anti-inflammatory actions[26,27,29,38-42]. Overproduction of the pro-inflammatory cytokine, IL-1β is thought to be involved in the perpetuation of inflammatory bowel disease, while IL-1ra, the naturally occurring antagonist of IL-1β is believed to counteract the actions of IL-1β; an imbalance of IL-1β and IL-1ra production is thought to be a major factor in the perpetuation of intestinal inflammation[27,38]. The production of IL-1ra is reported to be stimulated by several substances including adherent IgG, certain cytokines like IL-10[26,37,39,42,43]. IgGs adsorb to cellulose acetate carriers of Adacolumn (see below) and this may be one explanation for the elevation of IL-1ra during GMA. Similarly, IL-10 is reported to be a major Th2 cytokine that is known to inhibit the production of IFN-γ, IL-1β, IL-6, IL-8, IL-12 and TNF-α limiting the emergence of Th1 cytokine field[30,44]. In contrast, IL-6 is recently reported to have complex inflammatory actions. Thus it has a major role in leucocyte extravasation by activating endothelial cells and stimulating the production of chemokines including IL-8 and monocyte chemoattractant protein[15]. This indicates that circulating IL-6 can be a major pro-inflammatory factor. IL-18 has been recognized to be a potent inducer of IFN-γ production by T-cells[24,45]. Either independently or in synergy with IL-12, the effects of IL-18 via induction of IFN-γ can lead to a rapid activation of the monocyte/macrophage system with an upregulation of these cells' innate immune capabilities[46,47] that may lead to a Th1 type inflammatory response[23,24]. Hence high levels of circulating IL-18 should be considered pro-inflammatory.

One obvious question which has been drawing our attention in this study was why a consistent increase in IL-6 and IL-18 at the Adacolumn outflow could not be detected as was seen for IL-1ra and IL-10. It is not to say that they were not released from cells which adhere to the column carriers, rather to say that no increase was detected. However, it is logical to view the inside the column as an environment in which very high levels of active oxygen derivatives and proteases (released from adherent leucocytes) prevail. It is likely that the cytokines we have measured have different stability within the column environment and those which survive in appreciable amounts show an increase at the column outflow.

Patients with autoimmune diseases are known to have circulating immune complexes (ICs) in their plasma[49,50]. The Adacolumn adsorptive leucocytapheresis carriers are made of cellulose acetate and cellulose acetate is known to adsorb IgG and ICs from plasma[50]. Upon adsorption, the binding sites on IgG and ICs become available for the Fcγ receptors on neutrophils and monocytes/macrophages[50-53]. Further more, cellulose acetate with adsorbed IgG and ICs generates active complement fragments including C3a, C4a and C5a[5,50,53]. The opsonins, C3b, C3bi C4b, C4bi, C5b and others derived from active complement fragments also adsorb onto the carriers[5,50,53-55] and serve as binding sites for the leucocyte complement receptors, CR1, CR2, CR3 (Mac-1, CD11b/CD18) and CR4[54-57]. Leucocyte adsorption to the cellulose acetate carriers of Adacolumn is governed by the Fcγ receptors (FcγRs) and the complement receptors on the leucocytes.

The expression of these two sets of receptors is common on neutrophils and monocytes/macrophages. In contrast, the expression of complement receptors is not a common feature of lymphocytes except on small subsets of B, T and NK (natural killer) cells[55,58]. Similarly, FcγRs are not widely expressed on lymphocytes except on a small population of $CD19^+$B cells[58] and $CD56^+$NK cells[59]. These basic processes should explain why the carriers selectively adsorb granulocytes and monocytes/macrophages, but not lymphocytes.

In conclusion, elevated blood levels of IL-6 and IL-18 together with peripheral blood granulocytes and monocytes/macrophages which in patients with UC show activative behaviour and increased survival time can be pro-inflammatory and are not counteracted by anti-inflammatory cytokines during active UC. Depletion of these leucocytes by GMA was associated with remission of UC in most of the treated patients and a decline in circulating cytokines. These observations support the assumption that peripheral blood granulocytes and monocytes/macrophages influence the circulating levels of cytokines, hence UC disease and should be appropriate targets of the GMA therapy.

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CLINICAL RESEARCH

Non-alcoholic fatty liver disease may not be a severe disease at presentation among Asian Indians

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Abstract

AIM: To evaluate the clinical and biochemical profile of patients with non alcoholic fatty liver disease (NAFLD) and to assess their histological severity at presentation.

METHODS: Consecutive patients presenting to the liver clinic of All India Institute of Medical Sciences (AIIMS) with raised transaminases to at least 1.5 times upper limit of normal, and histologically confirmed non-alcoholic fatty liver disease were included. Patients who had significant alcohol intake or positive markers of other liver diseases or who were taking drugs known to produce fatty liver were excluded. The clinical, biochemical and histological profile of this group was studied.

RESULTS: Fifty-one patients with NAFLD formed the study population. Their median age and BMI were 34(17-58) years and 26.7(21.3-32.5) kg/m^2 respectively and 46 (90.1%) were males. The majority of the patients had mild inflammation, either grade 1 [32 (63%)] or grade 2 [16 (31%)] and only 3 (6%) patients had severe (grade 3) inflammation. Twenty-three (45%), 19 (37%), 8(16%) and 1(2%) patient had stage 0, 1, 2 and 3 fibrosis respectively on index biopsy and none had cirrhosis. On univariate analysis, triglyceride levels more than 150 mg % (OR = 7.1; 95% CI: 1.6-31.5, P = 0.002) and AST/ALT ratio > 1 (OR = 14.3; 95% CI: 1.4-678.5, P = 0.008) were associated with high grades of inflammation and none was associated with advanced fibrosis. On multivariate logistic regression analysis, hypertriglyceridemia >150 mg% was the only factor independently associated with presence of high grade of inflammation (OR = 1.6; 95% CI: 1.3-22.7, P = 0.02), while none was associated with advanced fibrosis. Triglyceride levels correlated positively with inflammatory grade (r = 0.412; P = 0.003).

CONCLUSION: NAFLD in North Indian patients is a disease of young over-weight males, most of whom are insulin resistant and they tend to have a mild histological disease at presentation.

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Key words: Inflammation; Fibrosis; Triglycerides; Non alcoholic steatohepatitis

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INTRODUCTION

With the increasing awareness and early recognition of asymptomatic patients with raised transaminases and the ongoing epidemic of the metabolic syndrome, non alcoholic fatty liver disease (NAFLD) has become a common cause of referral to hepatology clinics[1]. NAFLD can be divided into 4 histological types (types 1 to 4)[2]. Studies from the west have demonstrated that NAFLD, especially NAFLD type 3 and 4 can progress over a variable period, in 25%-40% patients, to cirrhosis and can contribute to liver-related mortality[2,3]. Recently there have been reports linking the development of hepatocellular carcinoma to non alcoholic steatohepatitis (NASH)[4,5]. Studies from Europe and United States have demonstrated that up to 40% patients with NAFLD have advanced fibrosis and a proportion even have cirrhosis on the index biopsy[6,7].

The prevalence of fatty liver in the general population of India has been shown to be as high as 24%[8], which is similar to that reported from some of the western countries, where it parallels the prevalence of obesity[9,10]. Despite the high prevalence of NAFLD in India and rapidly rising incidence of type 2 diabetes mellitus in this country, data on hepatic morphology, to indicate the severity of NAFLD is limited[11-13]. The above-mentioned 3 studies reported demographic and biochemical profile of patients with NAFLD. Histological parameters were studied in only a small number of patients. By now it is clear that histological type 3 and 4 NAFLD are

associated with progressive liver disease and therefore needs therapeutic interventions. The present prospective study was designed to assess the magnitude of advanced liver damage and fibrosis among consecutive patients with biopsy proven NAFLD.

MATERIALS AND METHODS

Study design

It is a cross sectional study to evaluate the clinical, biochemical and histological profile of patients with NAFLD. The study was approved by the ethics committee of the All India Institute of Medical Sciences(AIIMS), New Delhi.

Patients

Inclusion criteria: Consecutive patients presenting to the liver clinic of AIIMS, with raised transaminases to at least 1.5 times upper limit of normal, and histologically confirmed NAFLD, were included.

Exclusion criteria: Patients in whom alcohol intake exceeded 20 grams per day (history of alcohol intake was taken separately from the patients and the closest relatives), patients with positive markers for other liver diseases (hepatitis viruses A through E, autoimmune, Wilson's disease, alpha 1 anti-trypsin deficiency, hemochromatosis) and patients who were on medications known to induce fatty liver such as methotrexate, estrogens, amiodarone and tamoxifen were excluded.

Methods

All patients were evaluated with a thorough history and examination especially to exclude intake of significant amounts of alcohol and to exclude intake of potentially hepatotoxic drugs. Anthropometric measurements were taken at the initial visit. These included the weight, height, waist and hip circumferences. Body mass index (BMI) and waist hip ratios were calculated as weight/height^2. After an overnight fast, 10 mL of blood was collected for a complete blood count and biochemical investigations including a liver function profile, lipid profile, fasting serum insulin and fasting blood glucose.

Hepatitis virus serologies included hepatitis B surface antigen (HBsAg), total antibodies to hepatitis B core antigen (total anti-HBc) and antibodies to hepatitis C virus (anti-HCV). Anti-HCV was done using a sensitive commercial ELISA (Xcyton, Bangalore, India)[12] and the remaining viral serologies were done using commercial ELISA kits (Organon Teknika, Boxtel, The Netherlands). Serum ferritin and 24-h urinary copper estimation were done using conventional biochemical techniques[13,14]. Alpha one anti-trypsin (α1AT) phenotyping was done using isoelectric focusing (PHAST system; Pharmacia Biotech, Uppsala, Sweden)[15]. The anti-nuclear antibodies (ANA), anti- smooth muscle antibodies (ASMA) and anti-liver kidney and microsomal antibodies (anti-LKM1) were carried out using the immunofluorescence technique. Hepatitis C virus RNA was detected using the polymerase chain reaction method as standardized at our laboratory[15] .

Liver biopsy was done after an informed consent in all patients suspected to have NAFLD, using an 18-gauge Menghini's aspiration needle. Each biopsy specimen, after being fixed in 10% formalin, had routine hematoxylin-eosin stains and special stains such as reticulin, Masson's trichrome, Perl's iron stain and Orcein stain for copper and periodic acid Schiff after digestion with diastase. Immunoperoxidase staining to detect HBsAg and hepatitis B core antigen (HBcAg) was carried out in all patients to exclude the presence of chronic viral hepatitis. The classification given by Brunt *et al* was used to grade and stage NASH[16].

An ultrasonography scan of the upper abdomen was done in all patients in order to examine presence of fatty liver and to exclude any obstructive biliary pathology or the presence of any hepatic space occupying lesion.

Fasting serum insulin values were used along with simultaneous fasting blood glucose measurements to calculate HOMA-IR (homeostasis model assessment) according to the formula given below.

HOMA-IR = (fasting serum insulin (μIU/mL)×fasting plasma glucose (mg%))/(22.5 ×18)

Value of HOMA-IR more than 1.64 implied the presence of abnormally high insulin resistance[17].

Definitions

Diabetes was regarded to be present if the fasting venous plasma glucose was more than 126 mg%[18] . Patients with BMI of more than 23 were seen to be overweight and those with a BMI of >25 were labeled as obese (Asian standards)[19]. Hypertriglyceridemia was defined as a fasting serum triglyceride level of more than 150 mg%. Patients who had at least three of the following five components: hyperglycemia (fasting blood sugar >110 mg% or known type 2 diabetes mellitus, central obesity (as defined by a higher waist-hip ratio), hypertension, hypertriglyceridemia (serum triglyceride > 150 mg%), and low HDL cholesterol levels (< 50 for women and < 40 for men), were labeled to have the metabolic syndrome. Since we did not have waist hip ratio in a large number of patients, we used obesity (BMI > 25 kg/m^2) as a surrogate for high waist hip ratio, as one of the component of the metabolic syndrome[1,20].

Statistical analysis

The results were expressed as median (range) for continuous variables and as frequencies (proportions) for categorical variables. Factors suspected to influence the severity of grade and stage were tested in a univariate and multivariate logistic regression analysis. For the univariate and multivariate analyses, the dependent variable was the presence of mild inflammation (grades 0, 1) or severe inflammation (grades 2, 3) for assessing the association with inflammatory grade and mild fibrosis (stages 0, 1) or severe fibrosis (stages 2, 3, 4) for assessing association with fibrosis stage. The independent variables were age>35, sex, BMI > 25 kg/m^2, AST/ALT ratio >1, cholesterol >200 mg%, triglycerides >150 mg%, HOMA-IR >1.64, presence or absence of hypertension and metabolic syndrome. Correlation between histological severity and the above mentioned independent variables was carried out using Spearman's correlation. For skewed data, non-parametric tests were used wherever necessary. All data were analyzed using the SAS 8.0 statistical package.

RESULTS

From January 1999 to June 2005, 51 patients with histologically proven NAFLD were registered at the liver clinic of

AIIMS. This number formed 2.6% of all liver clinic referrals during this time period.

The median age of this cohort of patients was 34 (17-58) years, the BMI was 26.7 (21.3-32.5) kg/m^2 and 46 (90.1%) were males. Median AST/ALT ratio was 0.63 (0.31-2.61). Table 1 gives the baseline demographic and biochemical profile of the patients.

Prevalence of features of metabolic syndrome

Table 2 depicts the prevalence of individual features of the metabolic syndrome in these patients. Eighty percent patients had high HOMA-IR, while 69.4%, 40.8%, 36.4%, 11.8% and 10% had obesity, hypertriglyceridemia, low HDL cholesterol, hypertension and diabetes respectively. All the investigations and clinical features required for diagnosing the metabolic syndrome were available in 43 patients. Of these, 9(20.9%) patients fulfilled the criteria for a diagnosis of metabolic syndrome (presence of 3 or more components of the metabolic syndrome).

Histological severity and factors associated with severe disease

Twenty-eight (55%) patients had NASH (type 3 or 4 NAFLD) whereas the rest had only fatty liver with or without spotty necrosis without evidence of either necrosis or fibrosis (type 1 or 2 NAFLD).

Inflammatory grade: The majority of the patients had mild inflammation, either grade 1 [32 (63%)] or grade 2 [16 (31%)]. Only 3(6%) patients had severe or grade 3 inflammation.

We evaluated factors which could have been associated with moderate to severe inflammation (grade 2 or 3) versus those with mild inflammation (grade 0 or 1). On univariate analysis, triglyceride levels more than 150 mg % (OR = 7.1; 95% CI: 1.6-31.5, $P = 0.002$) and AST/ALT ratio > 1 (OR = 14.3; 95% CI: 1.4-678.5, $P = 0.008$) were significantly associated with high grades of inflammation. Presence of metabolic syndrome was more common among patients who had severe inflammation although it did not reach the significant value (Table 3). On multivariate logistic regression analysis, hypertriglyceridemia more than 150 mg% was the only factor independently associated with presence of high grade of inflammation on index biopsy (OR = 1.6; 95% CI: 1.3-22.7, $P = 0.02$). There was also positive correlation between the triglyceride levels and inflammatory grade ($r = 0.412$; $P = 0.003$) (Figure 1)

Fibrosis stage: Most of the patients had either no or minimal fibrosis. Twenty-three (45%) and 19 (37%) patients had stage 0 and stage 1 fibrosis respectively on liver biopsy. Eight (16%) had stage 2 and 1 (2%) had stage 3 fibrosis. No patient had cirrhosis on index biopsy. On evaluation of factors, which could have been associated with severe fibrosis (stages 2, 3, 4 *versus* stages 0,1), metabolic syndrome (OR = 5.6; 95% CI: 0.7-36.5, $P = 0.04$) and AST/ALT ratio > 1 ($P = 0.09$) were more commonly present among patients with advanced fibrosis but failed to reach significance (Table 4).

Table 1 Baseline demographic and biochemical parameters of the cohort

Parameters	Value
Age (yr)	
n	51
Median	34
(Range)	(16-58)
Sex ratio (males/females)	46/5
Body mass index (kg/m^2)	
n	49
Median	26.7
(Range)	(21.3-32.5)
Bilirubin (mg%)	
n	51
Median	0.8
(Range)	(0.5-13.0)
Aspartate aminotransferase (IU/L)	
n	51
Median	66
(Range)	(29-230)
Alanine aminotransferase (IU/L)	
n	51
Median	98
(Range)	(52-349)
Alkaline phosphatase (IU/L)	
n	51
Median	159
(Range)	(87-372)
Albumin (gm%)	
n	47
Median	4.6
(Range)	(3-5.4)
Cholesterol (mg%)	
n	49
Median	180
(Range)	(96-323)
Triglycerides (mg%)	
n	49
Median	145
(Range)	(48-339)
HDL cholesterol (mg%)	
n	44
Median	41
(Range)	(28-65)
Fasting plasma glucose (mg%)	
n	49
Median	90
(Range)	(70-150)
Fasting serum insulin (μIU/L)	
n	45
Median	10.4
(Range)	(1.6-28.4)
HOMA-IR	
n	45
Median	2.4
(Range)	(0.3-6.0)

HDL: High density lipoprotein, HOMA-IR: Homeostasis model assessment for insulin resistance.

DISCUSSION

Although NAFLD has been demonstrated to be present in almost one quarter of the general population[10] and has been

Table 2 Prevalence of individual components of metabolic syndrome

Component of metabolic syndrome	*n* (%)
Obesity	34 (69.4)
Hypertriglyceridemia	20 (40.8)
Low HDL cholesterol	16 (36.4)
High HOMA-IR	36 (80)
Hypertension	6 (11.8)
Diabetes	5 (9.8)
Metabolic syndrome	9 (20.9)

HDL: high density lipoprotein; HOMA-IR: Homeostasis model assessment for insulin resistance.

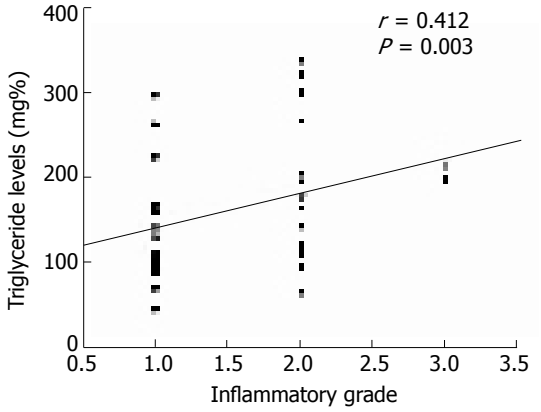


Figure 1 Scatter plot demonstrating positive correlation between triglyceride levels and inflammatory grade. [Coefficient of correlation (*r*) = 0.412; *P* = 0.003 using spearman's correlation].

Table 3 Factors associated with advanced grade of inflammation

Variables	Grade 0/1 (*n*/*N*)	Grade 2/3 (*n*/*N*)	*P*
Male	29/32	17/19	1.0
Age >35 years	13/32	10/19	0.40
BMI >25 kg/m^2	18/30	16/19	0.11
AST/ALT ratio > 1	1/32	6/19	0.008
Cholesterol > 200 mg%	9/30	5/19	1.0
Triglycerides >150 mg%	7/30	13/19	0.002
HOMA-IR >1.64	21/26	15/19	1.0
Hypertension	4/32	2/19	1.0
Metabolic syndrome	3/30	6/19	0.07

BMI: body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HOMA-IR: homeostasis model assessment for insulin resistance; Univariate analyses; *P* value on chi-square or Fisher's exact test.

Table 4 Factors associated with advanced stage of fibrosis

Variables	Stage 0/1 (*n*/*N*)	Stage 2/3/4 (*n*/*N*)	*P*
Male sex	4/42	1/9	1.0
Age >35 years	17/42	6/9	0.26
BMI >25 kg/m^2	26/42	8/9	0.24
AST/ALT ratio > 1	4/42	3/9	0.09
Cholesterol > 200 mg%	13/40	1/9	0.41
Triglycerides >150 mg%	14/40	6/9	0.13
HOMA-IR >1.64	28/36	8/9	0.66
Hypertension	5/42	1/9	1.0
Metabolic syndrome	5/40	4/9	0.04

BMI: body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HOMA-IR: Homeostasis Model assessment for insulin resistance; Univariate analyses; *P* value on chi-square and fisher's exact test.

shown to be responsible for one third of patients with asymptomatic transaminitis in India, little is known about the baseline clinical, biochemical and histological profile of these patients. The present study described the baseline profile in North Indian patients with biopsy proven NAFLD.

Our study demonstrated NAFLD to be a disease of younger age group with a mean age at presentation being 33.3 (8.01) years. More than 90% of the patients were males. This predilection for involvement of males has been seen not only in other studies from Northern India[13-15] , but also in the United States[21]. This is different from what used to be the classical phenotype of NASH patients, where it was described to be a disease of middle-aged females. The reason for this male preponderance has been described as a higher waist-hip ratio in men as compared to women, an indicator of central obesity and insulin resistance. Twenty-one percent of our patients with NAFLD were found to have the metabolic syndrome. The prevalence rate of metabolic syndrome in the general population of India has been reported to be approximately 8% in men older than 20 years[22], but no study has earlier reported the prevalence of the metabolic syndrome in patients with NAFLD from India.

The present study also highlights certain important differences between the phenotype of the cohort of NAFLD as seen in Northern India versus that which has been described classically from the west. This difference would not be surprising, considering the fact that the body fat distribution and risk of cardiovascular complications with similar amount of body fat differs between Asian Indians and their western counterparts. This has even prompted lowering of BMI and waist-hip ratio cut-offs for defining overweight and obesity among Asians[23,24]. Since obesity and fat distribution are integral parts of the insulin resistance syndrome and fatty liver forms part of the spectrum of abnormalities associated with this syndrome, the difference in the baseline profiles of NAFLD patients would be obvious.

The mean age of presentation of our patients, most of whom were detected during workup of asymptomatic transaminitis was about a decade younger than the western patients. The younger age of presentation may suggest increasing health awareness among our population with lower thresholds for health check-ups during which the asymptomatic transaminitis is picked up and investigated. It may also suggest the

epidemic proportion of the metabolic syndrome in India, wherein the population which is fast acquiring western lifestyle gets exposed to the detrimental effects of high calorie diet and physical inactivity at an earlier age. In fact, Indians have been shown to have higher body fat percentage and adverse pattern of body fat distribution including abdominal adiposity even when the BMI is within limits considered as normal for Caucasians[25]. Further, in the age group of 11-17 years, the prevalence of overweight and obesity is 11.63% and 2.38% in urban and 4.7% and 3.63% in rural Indian population respectively[26]. The mean BMI of our patients [26.5 (2.8) kg/m^2] was similar to cohorts described in other studies from North India[13,14], but significantly lower than what has been described in the Caucasian populations, where the mean BMI has always been reported to be above 30[2,3,8,26,27]. As described earlier, Indian patients despite having a lower BMI have similar body fat percentage as the Caucasians. This would mean higher visceral fat distribution, evidenced by higher waist hip ratios, despite lower BMIs. Unfortunately, waist hip ratios were not available in the present study for most of the patients. However, 80% of our patients had high HOMA-IR which is an index of insulin resistance and has been correlated earlier with visceral adiposity and higher waist hip ratios.

Most important finding in the present study was the presence of histologically mild disease in the cohort. Two thirds of the patients had minimal or mild inflammation (grade 0 or 1). Severe (grade 3) inflammation was present in only 3 patients. Hypertriglyceridemia was found to be significantly and independently associated with presence of severe inflammation.

It has already been demonstrated that insulin resistance leads to higher free fatty acid load to the liver, consequently higher triglyceride synthesis and increased secretion of triglyceride rich very low density lipoprotein (VLDL) from the liver[28,29]. In fact, circulating VLDL triglycerides have been correlated with liver fat density[30]. Higher triglyceride and fatty acid deposits may provide substrates for oxidative stress, which is a putative second hit in pathogenesis of progressive NAFLD[31]. High triglyceride levels have been shown to be independently associated with septal or advanced fibrosis by Ratziu *et al*. They also demonstrated that septal fibrosis had a significant positive correlation with liver necroinflammatory activity, thereby indirectly linking hypertriglyceridemia with necroinflammation[9]. In the present study however, we have demonstrated a direct correlation between hypertriglyceridemia and necroinflammatory activity in the liver.

Interestingly, none of our patients had cirrhosis on the index biopsy and only 2% had advanced (stage 3 or 4) fibrosis. Most (> 90%) had minimal or no fibrosis on presentation. Similar mild histology among NASH patients has been reported in other studies from India[13-15] as well as one study from Israel[32]. This is in contrast to the reported series from the west, where advanced fibrosis or cirrhosis has been demonstrated to be present in up to 50% of NAFLD cohorts[7-9,24]. One reason which can be cited for mild fibrosis in our patients is the younger age at presentation, because extent of fibrosis has been shown to increase with increasing age and increasing duration of disease[8,9]. Therefore it is possible that the cohort in the present study happened to have been detected at an early stage and with advancing age the disease might progress. This can only be confirmed later when follow up histology is available in these patients. Another factor which could be responsible for milder disease in these patients is the lower BMI. But this may not be true, because as has been discussed earlier, Asians with lower BMI may also be predisposed to similar risk of developing the metabolic syndrome as Caucasians with higher BMI. Further the effect of BMI or age was not seen on the degree of fibrosis in the univariate analysis. No other factor was found to be associated with severe fibrosis in the present study. The lack of association might be due to small number of patients in the group of severe fibrosis, as only a minority of patients had advanced fibrosis. Studies from the west, however have reported higher BMI, older age, type 2 diabetes mellitus, AST/ALT ratio > 1 and hypertriglyceridemia to be independent risk factors for presence of advanced fibrosis on index biopsy[8,9]. The milder histological disease in the present cohort may also represent a genetic predisposition in our patients, just like their genetic predisposition to develop metabolic syndrome. Whether these patients with mild fibrosis continue to have a mild disease or they progress to a stage of cirrhosis can only be answered once we have an adequate follow up on them.

To conclude, NAFLD in North Indian patients is a disease of young overweight males, most of whom are insulin resistant and they tend to have a mild histological disease at presentation.

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RAPID COMMUNICATION

Perinuclear anti-neutrophil cytoplasmic antibodies (p-anca) in chronic ulcerative colitis: Experience in a Mexican institution

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Abstract

AIM: To assess the prevalence and clinical value of p-ANCA in a sample of Mexican ulcerative colitis (UC) patients.

METHODS: In a prospective, IRB-approved protocol, p-ANCA was determined in 80 patients with UC (mean age, 32 ± 12.9 years). The severity and extension of disease were determined by clinical methods, searching a statistical association with p-ANCA status.

RESULTS: p-ANCA were detected in 41 (51%) patients. Severity of disease was the only clinical variable statistically associated with their presence ($P < 0.0001$; OR = 9; CI 95% = 3.2-24.7).

CONCLUSION: The prevalence of p-ANCA was similar to that reported in other countries. Their presence was associated to UC severity, but offered no more information than the obtained by clinical methods.

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Key words: Ulcerative colitis; Inflammatory bowel disease; Perinuclear anti-neutrophil cytoplasmic antibodies

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INTRODUCTION

Anti-neutrophil cytoplasmic antibodies (ANCA) have been described in subjects with different types of vasculitis, and they are essential to establish the diagnosis of Wegener´s granulomatosis, microscopic polyangiitis, Churg-Strauss Syndrome and renal-limited vasculitis[1-4]. Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA), a subset of ANCA, have been described in patients with inflammatory bowel disease, mainly ulcerative colitis (UC). However, their role in pathogenesis and diagnostic value are still controversial[5]. Previous reports have suggested that p-ANCA might be a sensitive and specific test to diagnose UC[6], to distinguish it from Crohn's disease and other colitides[7], and to provide a prognosis regarding response to medical treatment and risk of pouchitis following the pelvic pouch[8,9].

The prevalence of p-ANCA in patients with UC varied from 40% to 88%[10-13]. High titers have been reported by some authors in subjects with active disease, but its clinical value is controversial because others have not found any correlation between their levels and the activity or extension of disease[14-18].

The value of a diagnostic test, such as p-ANCA, should be evaluated by several practical factors like costs, availability, and usefulness for clinical decisions before accepting them as a standard diagnostic tool.

The aim of this study was to assess the prevalence and clinical usefulness of p-ANCA in a sample of Mexican patients with UC.

MATERIALS AND METHODS

A prospective study was performed at the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, which is a referral center for gastrointestinal diseases in Mexico City. The protocol was reviewed and approved by the Institutional Review Board. All patients invited to participate in the study signed an informed consent before inclusion. Eighty consecutive patients with diagnosis of UC were included. In all cases, the diagnosis of UC was based on clinical, endoscopic and histopathologic findings. The extension of disease was determined by total colonos-

copy, and categorized into pancolonic or left-sided colitis. The severity of disease was determined on clinical basis according to the Truelove and Witts criteria[19], and categorized into mild, moderate and severe. Clinical data, including severity and extension of disease were collected by an investigator who was blind to the results of p-ANCA.

p-ANCA determination

Detection of ANCA was done by Indirect Immunofluorescence (IFL), in accordance with the recommendation of the International Workshop[20]. Human neutrophils were prepared from peripheral blood of patients and normal healthy volunteers. The slides were fixed in 98% ethanol at 4℃ for five minutes and dried quickly in air. After using phosphate-buffered saline (PBS) in a dilution of 1:40, the elements where dissolved twofold until it reached 1:320. Following incubation for 1 h r at RT, the slides were washed three times with PBS and bound antibodies were incubated for 30 min and detected with FITC-conjugated rabbit anti-human IgG at RT. Subsequently, the slides were washed three times with PBS and covered with glycerin-phosphate-buffered saline. A titer of 1:20 or higher was considered positive.

The antigen-specific enzyme-linked immunosorbent assay (ELISA) method was used to test the sera for the presence of either mieloperoxidase (MPO) or anti-proteinase-3 antibodies. Human MPO or human proteinase-3 were diluted to 2 mg/L in carbonate buffer (pH 9.6) and 100 μL of each solution was placed in the wells of a 96-well microplate and left for 24 h at 4℃. After plate was washed with PBS three times, 100 μL of diluted sera (1:100 in PBS containing 0.1% tween 20 and 0.1% skim milk) was added to each well, and the plate incubated for 1 h at 37℃. After washing, the enzyme reaction was performed, and color development was measured with a microplate colorimeter. Optical density values > 2 SD more than the mean of the control subjects were considered positive.

Statistical analysis

The student t- -test was used for dimensional variables, and the association between categorical variables was studied by means of the χ^2 with Yates correction. The *P* values were 2-tailed and less than 0.05 was considered statistically significant.

RESULTS

Eighty patients with diagnosis of UC were included. There were 41 men and 39 women, with a mean age of 32 ± 12.9 years. The mean duration of disease was 7.2 ± 6.6 years. Pancolitis was present in 58% and left-sided colitis in 42%. Disease severity was categorized as severe in 20 (25%), moderate in 7 (10%), mild in 15 (18%), and there was no evidence of activity in the remaining 38 (47%) patients. Extraintestinal manifestations were present in 43%; and corresponded to arthritis (23%), primary sclerosing cholangitis (7.5%), erythema nodosum (5%), ankylosing spondylitis (2.5%), pyoderma gangrenosum (2.5%), anterior uveitis (2.5%), and aphthous ulcers (2.5%). Fourteen (25%) patients required surgical treatment due to failure of medical therapy.

Table 1 Clinical features in UC patients with positive and negative p-ANCA

Clinical feature	p-ANCA + *n* = 41	p-ANCA - *n* = 39	*P* value	OR	CI 95%
Pancolitis	22	24	0.62	0.72	(0.29-1.76)
Left-sided	19	15			
Colectomy	7	7	0.84	0.94	(0.29-2.98)
No colectomy	34	32			
EIMs present	14	17	0.52	0.67	(0.27-1.65)
EIMs absent	27	22			
Severe	31	10	< 0.0001	9	(3.2-24.7)
No Severe	10	29			

OR = odds ratio; CI 95% = Confidential interval 95%; *n* = Number of patients.

The p-ANCAs were detected by IFL in 41 (51%) and c-ANCA in 4 (5%) subjects. Titers ranged from 1:20 to 1:160. The antigenic specificity of p-ANCA tested by ELISA in 33 patients showed a positive reaction for myeloperoxidase in 29 and proteinase-3 in 4. Age, gender and age at diagnosis was similar between patients with or without p-ANCA .

A statistical association was observed between p-ANCA status and the severity of UC (*P* = < 0.0001; OR 9, CI 3.2-24.7). Twenty-two (48%) patients with pancolitis and 19 (56%) with left-sided colitis were positive for p-ANCA (*P* = 0.47; OR = 0.72; 95% CI: 0.27-1.94). Seven out of the 14 (50%) patients who underwent surgical treatment were positive as compared with 34 of 66 (52%) who were not operated upon [*P* = 0.91; OR = 0.94; 95% CI: 0.26-3.34].No association was found between the presence of extraintestinal manifestations and p-ANCA. See Table 1.

DISCUSSION

The determination of p-ANCA could give information in subjects with UC in three different clinical issues: (1) as a marker of genetic heterogeneity; (2) to assess inflammatory activity, and (3) for prognosis regarding response to medical treatment and postoperative outcome (pouchitis).

The prevalence of p-ANCA in our series was 51%, within the range published by other authors in different parts of the world[9-13]. It supports that not all patients with UC exhibit the same immunologic pattern, a fact against a pathogenic role of ANCA's in subjects with UC. On the other hand, the wide range of prevalence reported in the literature that goes from 40 to 88% could be rather explained by ethnic or genetic variations than by technical difficulties since both, IFL and ELISA, are simple, sensible, and reproducible assays, as observed in the present study[14,21].

Ethnic and genetic variability have been consistently reported in patients with IBD[22,23]. In a previous study, the authors found that Mexican patients with UC had an increased frequency of HLA-DR1 (DRB1*0102 and 0103) and HLA-DR2 (DRB1*15) when compared with healthy controls. HLA-DRB1*0103 and *0102 were associated

with more severe disease and necessity of surgical treatment[24]. Yang *et al* observed a linkage between p-ANCA positive UC patients and HLA-DR2[25]. The clinical usefulness of p-ANCA as a genetic marker was suggested many years ago by Shanahan who found a higher prevalence of p-ANCA in first-degree relatives of patients with UC[26]. This finding was confirmed by Lee[27] *et al*, in a different ethnic group. So, p-ANCA determination could be useful as a marker of genetic predisposition. However, today there is no information about predictive values of p-ANCA in this setting, and more research is necessary to answer this important question.

Several studies, although not all, have demonstrated a correlation between serum levels of p-ANCA and severity of UC[5,12,15,29-31]. In the present study, an association between the presence of p-ANCA and a subset of patients with severe UC was found but, as other studies, there was no correlation between levels of p-ANCA and the activity of disease evaluated by clinical and biochemical methods. From the point of view of the authors, a sophisticated laboratory exam such as the p-ANCA determination may not currently have a role in the evaluation of severity because it can be evaluated by simple and reproducible methods.

Fleshner *et al*, reported high pre-operative levels of p-ANCA in subjects who developed chronic pouchitis after the ileo-anal pouch[8]; however, it should be noted that some patients with acute and chronic pouchitis were p-ANCA negatives. The follow-up of patients in our series is still too short to analyze this aspect.

In conclusion, the prevalence of p-ANCA in this series was similar to that reported in other countries. Their presence was associated to severity of UC, but offered no more information than the obtained by clinical methods. Future research may clarify their role as a screening test for the first-degree relatives of UC patients, or for patients requiring an ileo-anal pouch.

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RAPID COMMUNICATION

Experimental obstructive jaundice alters claudin-4 expression in intestinal mucosa: Effect of bombesin and neurotensin

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Abstract

AIM: To investigate the influence of experimental obstructive jaundice and exogenous bombesin (BBS) and neurotensin (NT) administration on the expression of the tight junction (TJ)-protein claudin-4 in intestinal epithelium of rats.

METHODS: Forty male Wistar rats were randomly divided into five groups: I = controls, II = sham operated, III = bile duct ligation (BDL), IV = BDL+BBS (30 μg/kg per d), V = BDL+NT (300 μg/kg per d). At the end of the experiment on d 10, endotoxin was measured in portal and aortic blood. Tissue sections of the terminal ileum were examined histologically and immunohistochemically for evaluation of claudin-4 expression in intestinal epithelium.

RESULTS: Obstructive jaundice led to intestinal barrier failure demonstrated by significant portal and aortic endotoxemia. Claudin-4 expression was significantly increased in the upper third of the villi in jaundiced rats and an upregulation of its lateral distribution was noted. Administration of BBS or NT restored claudin-4 expression to the control state and significantly reduced portal and aortic endotoxemia.

CONCLUSION: Experimental obstructive jaundice increases claudin-4 expression in intestinal epithelium, which may be a key factor contributing to the disruption of the mucosal barrier. Gut regulatory peptides BBS and NT can prevent this alteration and reduce portal and systemic endotoxemia.

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Key words: Obstructive jaundice; Tight junctions; Claudin-4; Intestinal permeability; Intestinal barrier; Regulatory peptides; Bombesin; Neurotensin

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INTRODUCTION

Patients with obstructive jaundice, especially when exposed to the additional stress of an invasive diagnostic or therapeutic procedure, are prone to septic complications and renal dysfunction contributing to high morbidity and mortality rates[1]. Systemic endotoxemia appears to play a key role in the development of these complications[2]. Experimental and clinical studies have shown that obstructive jaundice increases intestinal permeability permitting the escape of endotoxin from the gut lumen[3,4]; however, little is known of the molecular events leading to intestinal permeability alterations.

Tight junctions (TJs) are located at the apical part of lateral membranes of polarized epithelial and endothelial cells forming a barrier that regulates the permeability of ions, macromolecules and cells through the paracellular pathway[5,6]. Up to now, three groups of macromolecules are considered as bona fide integral components of the tight junction: occludin, claudins and junctional adhesion molecule[6]. We have recently demonstrated using immunohistochemistry that intestinal mucosal barrier dysfunction in obstructive jaundice is associated with regional loss of occludin expression in the intestinal epithelium, observed mainly in the upper part of the villi[7]. Our findings were recently confirmed by other

investigators using immunoblotting[8].

Claudins comprise a multigene family consisting of 24 members believed to be major functional elements of TJ[9]. TJ strands are copolymers of heterogeneous claudin species and occludin, and heterogeneous claudin species constitute the backbone of TJ strands *in situ*[10]. The localization and contribution to barrier function of each member of the claudin family vary among tissues. Claudin-4 is highly expressed in rat intestine[11], with a preferential localization in the epithelium overlying the tips of intestinal villi and the follicle associated epithelium (FAE) of gut associated lymphoid tissue (GALT)[12]. We focused on this molecule because in obstructive jaundice loss of occludin expression was observed mainly at the upper part of the villi where claudin-4 is preferentially expressed.

Regulatory peptides bombesin (BBS) and neurotensin (NT), with a wide spectrum of biological actions on gastrointestinal tissues (influencing intestinal growth and adaptation, intestinal motility, blood flow, secretion, nutrient absorption and immune response), exert a protective role in preserving gut barrier integrity after various injurious insults[13-18]. Beyond their potent enterotrophic action we have shown that BBS and NT fully restored intestinal occludin expression in bile duct ligated rats, thus preventing endotoxin translocation. It suggests a role of these peptides as molecular modulators of TJs[7].

The present study was undertaken to investigate the effect of experimental obstructive jaundice on intestinal claudin-4 expression and examine the potential effect of BBS and NT on this parameter of intestinal mucosal barrier.

MATERIALS AND METHODS

Animals

Forty male albino Wistar rats, weighing 250-320 g, were used in the study. They were housed in stainless-steel cages, three rats per cage, under controlled temperature (23℃) and humidity conditions, with 12-h dark/light cycles, and maintained on standard laboratory diet with tap water *ad libitum* throughout the experiment, except for an overnight fast before surgery.

The experiments were carried out according to the guidelines set forth by the Ethics Committee of Patras University Hospital, Patras, Greece.

Experimental design

Animals were randomly divided into five groups: I (n = 8), controls; II (n = 8), sham operated; III (n = 8), bile duct ligation (BDL); IV (n = 8), BDL and BBS treatment and V (n = 8), BDL and NT administration. All surgical procedures were performed under strict sterile conditions, and light ether anesthesia. Rats from groups II, III, IV and V underwent laparotomy on d 0. *Via* a 1 cm upper midline incision, the gastroduodenal ligament was isolated and the common bile duct was mobilized. In groups III, IV and V, the common bile duct was further doubly ligated in its middle third with a 4-0 silk suture and was transected between the two ligatures. The abdominal incision was closed in two layers with chromic 4-0 cat gut and 4-0 silk.

For the 10 subsequent days, the animals of groups IV and V were treated daily with BBS (10 μg/kg, subcutaneously, three times a day) and NT (300 μg/kg, intraperitoneally, once a day) respectively, while the animals of groups I, II, and III were divided to receive daily either three subcutaneous or one intraperitoneal injection of 0.5 mL normal saline. All injections were given after topical application of an antiseptic solution of povidone iodine 10%. Previous studies have shown that the way of saline administration does not affect the results[7]. On the 10th d, all animals were operated (group I) or reoperated (groups II, III, IV and V). Samples were obtained according to the experimental protocol, after which the rats were sacrificed by exsanguination.

Peptides preparation

Bombesin: A stock solution of BBS (Sigma Chemical Co, St. Louis, Missouri, USA) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 1% (w/v) bovine serum albumin, so that the amount of BBS needed for each injection to be contained in a volume of 0.1 mL. The solution was divided into equal aliquots of 0.1 mL which were stored in plastic tubes at -20℃. At the time of administration, in order to prolong absorption, each aliquot was mixed with 0.4 mL of a solution 8% (w/v) hydrolyzed gelatin (Sigma Chemical Co, St. Louis, Missouri, USA). A final volume of 0.5 mL, containing 10 μg BBS/kg body weight, was injected subcutaneously three times daily. Selection of dose and route of administration was based on previous reports[7].

Neurotensin: A stock solution of NT (Sigma Chemical Co, St. Louis, Missouri, USA) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 0.1% (w/v) bovine serum albumin, so that the amount of BBS needed for each injection to be contained in a volume of 0.1 mL. The solution was divided into equal aliquots of 0.1 mL which were stored in glass vials at -20 ℃. At the time of administration, each aliquot was further diluted with 0.4 mL sterile saline to a final volume of 0.5 mL and was given intraperitoneally through a bolus injection containing 300 μg NT/kg body weight. Selection of dose and route of administration was based on previous reports[7].

Bilirubin and endotoxin measurements

For the determination of total bilirubin, 0.5 mL of blood was collected from all animals, by transecting the tip of their tail. Then a laparotomy was performed and in all groups, the portal vein and the abdominal aorta were punctured and samples of 1 and 2 mL of blood, respectively, were obtained for estimation of endotoxin. Bilirubin concentrations were assayed using a standard biochemical technique and expressed in mg/dl. Endotoxin concentration was determined by the quantitative chromogenic Limulus Amebocyte Lysate test (QCL-1000, BioWhittaker, Walkersville, USA) and expressed in EU/mL. Samples were processed according to the manufacturer's instructions. By this test it is possible to measure concentrations of endotoxin $\geqslant$ 0.01 EU/mL.

Immunohistochemistry for claudin-4

In tissue sections of the terminal ileum from all rats a standard immunohistochemical technique was applied to detect claudin-4. Deparaffinization, rehydration and microwave antigen retrieval were performed in Trilogy™ solution (Cell Marque, Hot Springs, AK). Then slides were incubated for 1 h at room temperature with a commercially available mouse monoclonal anti-claudin-4 antibody (1:40, ZYMED Laboratories, San Francisco, CA), followed by antigen-antibody detection using the DAKO ChemMate EnVision detection kit (DAKO A/S, Glostrup, Denmark). Colour was developed with diaminobenzidine (Sigma Fast DAB tablets, D-4293, St. Louis, Mo) and counterstained with Harris hematoxylin. All procedures took place at room temperature and between steps sections were washed in TBS. In negative control slides, the primary antibody was substituted by mouse serum. Twenty randomly selected adjacent perpendicularly sectioned villi from each case (n = 8 cases per group) were evaluated. The frequency of claudin-4 immunohistochemical expression in the upper third of the villi was recorded by dividing the number of claudin-4 positive enterocytes by the total number of enterocytes in the upper third of each villous.

Statistical analysis

The results are expressed as mean (SD). Comparisons among multiple groups were performed using the one-way ANOVA, followed by Dunnette's T3 post hoc test. Variance equality was tested by Levene statistical analysis. Differences were considered significant when $P < 0.05$.

RESULTS

Animal outcomes

All animals survived and were in good health throughout the experiment. Bile duct ligated rats were clinically jaundiced within three days. At reoperation on d 10 it was found that the ligation and division of the common bile duct in groups III, IV and V had been successful in all cases and resulted in dilatation of the common bile duct remnant proximal to the ligature without signs of bile leakage.

Bilirubin and endotoxin concentrations

Bile duct ligation led to significantly raised total bilirubin levels in groups III, IV and V compared with control and sham groups ($P < 0.001$). Figure 1 demonstrates endotoxin values, measured in blood collected from portal vein and aorta. Jaundiced animals (group III) presented significantly elevated endotoxin concentrations in portal and aortic blood compared with groups I and II ($P < 0.001$). When ligation of common bile duct was followed by either BBS or NT treatment, endotoxin values were significantly reduced both in portal vein ($P < 0.001$, respectively) and aorta ($P < 0.001$, respectively).

Evaluation of histology and claudin-4 immunostaining

Overall, the ileal architecture remained intact and epithelial continuity was retained, in all specimens studied, while no significant pathology was observed. Obvious alterations of claudin-4 immunohistochemical expression in the intestinal epithelium were observed among experimental groups. In control and sham operated rats claudin-4 was expressed in a few villous surface epithelial cells (Figure 2A), while occasionally unstained villi were observed. Claudin-4 was detected as membranous immunostaining, stronger on the basolateral sides than on the apical sides, with weak cytoplasmic staining (Figure 2A). In jaundiced rats, claudin-4 was expressed in most epithelial cells lining throughout the upper third of the villi, and this staining was observed in every villous (Figure 2B). Also the lateral expression of claudin-4 was upregulated in jaundiced rats (Figure 2D). In BBS or NT-treated rats claudin-4 expression was restored to the control state (Figure 2C). The frequency of claudin-4-positive cells in the upper third of the villi was significantly increased in jaundiced rats as compared with control and sham operated ($P < 0.001$), whereas it was reduced in rats administered BBS or NT ($P < 0.001$ as compared to BDL) (Figure 3).

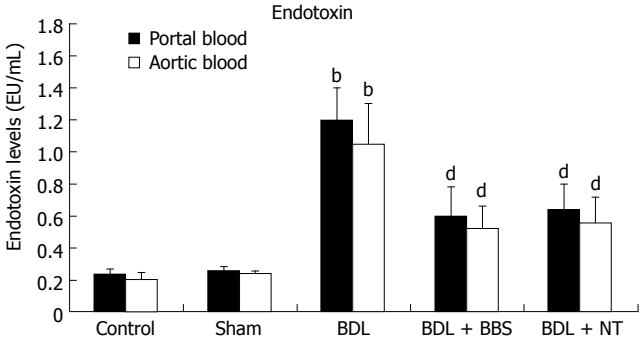


Figure 1 Portal and aortic endotoxin concentrations. Values are mean ± SD, $^{b}P < 0.001$ *vs* sham; $^{d}P < 0.001$ *vs* BDL. BDL, bile duct ligation; BBS, bombesin; NT, neurotensin.

DISCUSSION

Increased intestinal permeability has been postulated to be a key factor contributing to bacterial and endotoxin translocation and the pathogenesis of the so called "gut derived sepsis"[19]. In obstructive jaundice, increased intestinal permeability has been demonstrated not only in animal models but in patients as well and has been positively correlated with severity of biliary obstruction[3,4]. The present study, using an experimental model of biliary obstruction for 10 d in rats, demonstrated significant portal and aortic endotoxemia, a clinically important marker of ineffective intestinal barrier, accompanied by altered immunohistochemical expression of the TJ-protein claudin-4 in intestinal epithelium.

The molecular mechanisms responsible for permeability alterations in obstructive jaundice have not been adequately investigated until now. Previously we had shown for the first time that intestinal mucosal barrier dysfunction in obstructive jaundice is associated with regional loss of expression of the TJ-protein occludin in the intestinal epithelium, observed mainly at the upper part of the villi[7]. Our immunohistochemical observations were confirmed with immunoblotting method by other investi-

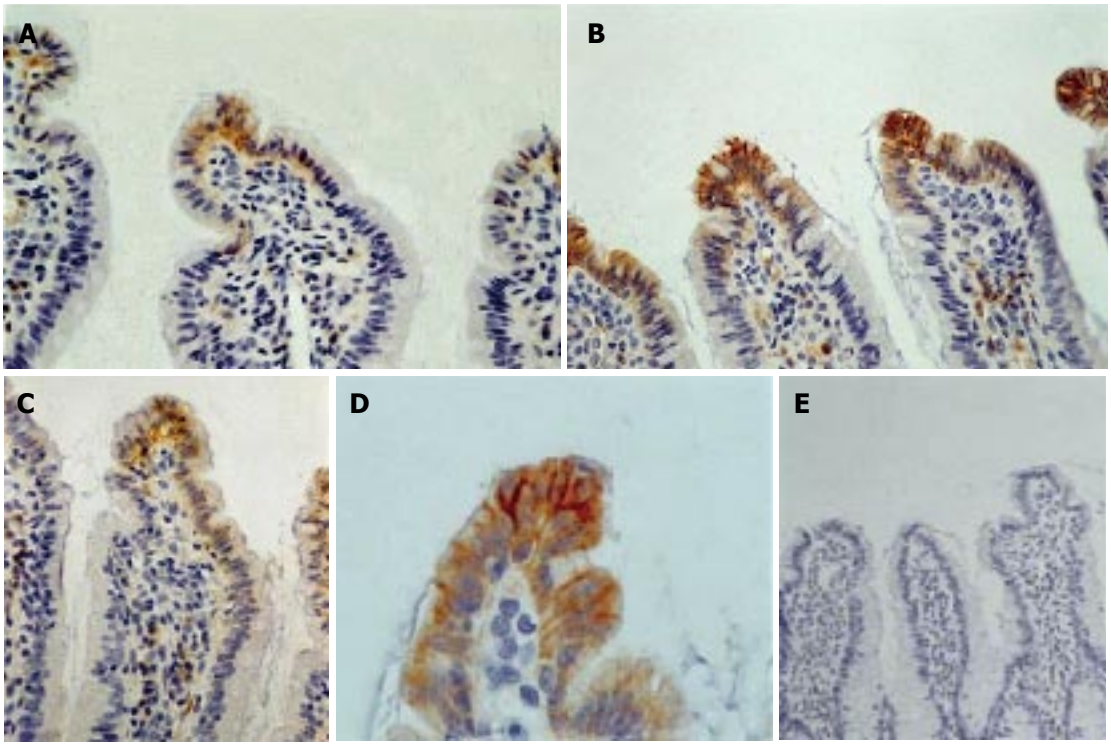


Figure 2 Immunohistochemical expression of claudin-4 in the mucosa of terminal ileum: In control and sham operated rats claudin-4 is expressed on the basolateral sides of a few villous surface epithelial cells (**A**: original magnification x 400). In obstructive jaundice, immunoreactivity for claudin-4 is stronger and this molecule is expressed by most epithelial cells lining throughout the upper third of the villi and not only in the tip (**B**: original magnification x 400). At higher magnification we can see the enhancement of the lateral distribution of claudin-4 in obstructive jaundice (**D**: original magnification x 1000). In bombesin or neurotensin-treated rats claudin-4 expression is restored to the control level (**C**: original magnification x400). Substituting anti-claudin-4 antibody by mouse serum no staining is observed (**E**: original magnification x 200).

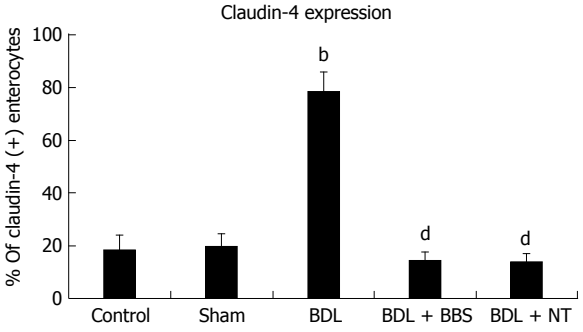


Figure 3 Frequency of claudin-4 (+) enterocytes in the upper third of the villi. Values are mean ± SD, $^{b}P < 0.001$ *vs* sham; $^{d}P < 0.001$ *vs* BDL. BDL, bile duct ligation; BBS, bombesin; NT, neurotensin.

gators, who additionally showed that obstructive jaundice led to decreased mucosal expression of the TJ-associated protein zonulin-1 as well[8]. The present study offered further insight into TJs alterations in the intestinal mucosa in obstructive jaundice, demonstrating upregulation of claudin-4 expression in the upper part of the villi.

The results obtained by the present study may initially seem paradoxical and a question raised is how increased expression of a TJ-protein may lead to increased permeability. Claudins are the only known variable elements in TJs and different expression, combination and mixing ratios of various members of the claudin family are essential in the regulation of barrier properties of TJs[20]. Overexpression of some claudins is often associated with the expected reduction of permeability, e.g. claudin-1 overexpression in Madin-Darby canine kidney (MDCK) cells increases transepithelial resistance[21]. On the other hand, increased blood-brain barrier permeability in inflammatory pain is associated with significant increase of claudin-3 and -5 expression, while occludin expression is decreased[22]. Regarding the functional role of claudin-4 in the intestinal epithelium, it may be associated with loosening of intercellular junctions and opening of the paracellular route, as indicated by findings of previous studies[12]. For instance, it has been shown that there is a close association between the locations of claudin-4 and TUNEL-positive apoptotic cells in intestinal villi and the FAE of GALT, suggesting that claudin-4 may be involved in the process of peeling off epithelial sheets of apoptotic enterocytes. In addition, claudin-4 is preferentially expressed at the apices of intestinal villi and the FAE, which represent important antigen-sampling sites. Given that both apoptosis and antigen capturing are associated with looseness of intercellular junctions, Tamagawa *et al* suggested that claudin-4 expression contributes to the formation of relatively loose intercellular junctions[12]. The present study provides support to the above proposal. Furthermore, taking into consideration our recent findings of decreased intestinal occludin expression in obstructive jaundice[7] and the results of the present study showing increased intestinal claudin-4 expression, one could hypothesize the existence of a novel "switch mechanism" by which claudin-4 may replace occludin and lead to increased permeability of tight junctions. The key role of claudin-4 and occludin in obstructive jaundice-associated intestinal permeability alterations is further evidenced by improvement of gut mucosal barrier

after restoration of their expression by regulatory peptides administration.

Which is the critical factor inducing these alterations in obstructive jaundice? The present study cannot answer this question due to the interplay of several factors in the intact organism. However, a recent *in vitro* study has shown that bile is crucial for maintenance of intestinal TJs integrity[8]. Beyond absence of intraluminal bile other factors such as systemic endotoxemia, cytokinemia and intestinal oxidative stress may contribute to intestinal barrier failure in obstructive jaundice[5,23-25]. Oxidative stress, attracting a growing research interest, has been recognized as an important modulator of TJs. It has been shown that oxidative stress increases epithelial permeability through modulation of the assembly, localization, expression and function of the molecular components of the TJ structural complex[24]. We have recently shown that experimental obstructive jaundice induces intestinal oxidative stress, demonstrated by increased lipid peroxidation, protein oxidation and oxidation of non-protein and protein thiols[25]. Therefore, oxidative stress may be implicated in altered occludin and claudin-4 expression in the intestinal mucosa of jaundiced rats.

The present study also showed that when jaundiced rats were treated with either BBS or NT, claudin-4 expression in the intestinal epithelium was restored to the control state and endotoxin levels in portal and aortic blood were significantly reduced. These regulatory peptides may have restored claudin-4 expression via a direct receptor-mediated mechanism. Animal studies provide evidence for the presence of BBS and NT receptors in intestinal epithelial cells[26,27]. Binding to peptides' specific G protein-coupled receptors activates multiple intracellular signals including protein phosphorylation pathways, accumulation of cyclic-adenosine monophosphate and mobilization of intracellular Ca^{++}[28], which may account for the maintenance of structurally and functionally effective TJs[6,20]. A potential molecular mechanism mediating the effect of BBS and NT on claudin-4 expression is activation of protein kinase C (PKC). PKC plays a central role in transducing neuropeptides' signals[28] and recent studies have shown that PKC activity is required for normal claudin assembly and the integrity of the intestinal epithelial barrier[29]. Also the antiapoptotic effect of BBS and NT on the intestinal epithelium of jaundiced rats[25] may be related to their modulating action on the expression of claudin-4, since there is evidence that intestinal apoptosis and claudin-4 expression may be interconnected[12]. Additionally, indirect mechanisms such as reduction of systemic endotoxemia, improvement of energy supply to enterocytes through splachnic vasodilatation[30,31] and reduction of intestinal oxidative stress[25] may contribute to the regulatory action of BBS and NT on the TJ structure and function.

In conclusion, our results show that enhanced claudin-4 expression in the upper part of the intestinal villi represents an important molecular mechanism for increased intestinal mucosal permeability in obstructive jaundice. Gut regulatory peptides BBS and NT, acting as molecular modulators of TJs, totally restored this alteration thus preventing endotoxin translocation from the gut lumen. These findings offer further insight into the pathophysiology of gut barrier failure in obstructive jaundice and suggest the potential therapeutic efficacy of regulatory peptides. However, further investigations to elucidate the details of the functional mechanisms and possible side effects of BBS and NT are needed before their clinical application.

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RAPID COMMUNICATION

Role of soluble triggering receptor expressed on myeloid cells in inflammatory bowel disease

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Abstract

AIM: To investigate the probable role of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) in the pathogenesis of inflammatory bowel disease (IBD).

METHODS: Fifty-eight patients were enrolled; nineteen healthy volunteers served as controls; 8 patients were diagnosed with Crohn's disease, and 31 with ulcerative colitis. Clinical and endoscopic activity indexes of patients with Crohn's disease and ulcerative colitis respectively were estimated. Upon admission blood was sampled; sTREM-1 and TNFα were measured by an immunoassay and malondialdehyde (MDA) by the thiobarbiturate assay, after passage through an HPLC system.

RESULTS: Median ± SE of TNFα of controls, patients with Crohn's disease and patients with ulcerative colitis were 6.02 ± 3.94, 7.98 ± 5.08 (P = NS *vs* controls), and 8.45 ± 4.15 ng/L (P = 0.018 *vs* controls) respectively. Respective values of sTREM-1 were 53.31 ± 32.93, 735.10 ± 197.17 (P = 0.008 *vs* controls) and 435.82 ± 279.71 ng/L (P = 0.049 *vs* controls). sTREM-1 was positively correlated with Crohn's disease activity index and clinical and endoscopic activity indexes of ulcerative colitis (P = 0.002, 0.001 and 0.009, respectively). sTREM-1 of patients with ulcerative colitis was positively correlated with TNFα (P = 0.001).

CONCLUSION: sTREM-1 seems to behave as a novel mediator in IBD in correlation with the degree of the inflammatory reaction of the intestinal mucosa.

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Key words: sTREM-1; Pro-inflammatory cytokines; Malondialdehyde; Inflammatory bowel disease

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INTRODUCTION

Accumulated evidence over the last decade demonstrates that inflammatory bowel disease (IBD) is a heterogeneous group of diseases resulting from different pathogenetic mechanisms with a common symptomatic expression[1]. Dysfunction of the innate and adaptive immune systems associated with gut mucosa might involve impairment of mucosal barrier function and development of localized or systemic inflammatory and autoimmune processes[2,3]. Triggering receptor expressed on myeloid cells (TREM)-1 is a recently discovered receptor expressed on the surface of neutrophils and monocytes in the presence of microbial components. Engagement of TREM-1 has been reported to trigger the synthesis of proinflammatory cytokines, though its expression failed to elevate in several autoimmune diseases[4,5]. Expression of TREM-1 was not identified on cell membranes of macrophages of intestinal lamina propria; its absence was connected with prevention of excessive inflammatory reactions by bacterial flora, and thus, of any tissue damage of the intestine[6].

A soluble form of TREM-1, named sTREM-1, has been found; it is thought to be released from cell membranes as a result of the severity of inflammation. sTREM-1 was found elevated in samples of gastric juice of patients with peptic ulcer disease; its correlation with gastritis score leads to the assumption of a probable implication of sTREM-1 in the pathogenesis of gastritis[7]. Based on the latter findings in gastric diseases, the present study aimed to clarify the significance of sTREM-1, if any, in inflammatory bowel disease.

MATERIALS AND METHODS

Subjects

Fifty-eight patients were enrolled in the prospective

study from January 2001 to June 2001; nineteen healthy volunteers served as controls; eight patients were diagnosed with Crohn's disease; and 31 with ulcerative colitis. Exclusion criteria for the study were (1) gastric or colonic neoplasia, (2) liver cirrhosis, (3) any history of administration of monoclonal anti-TNFα antibodies (infliximab, adalimubab) or soluble TNFα receptors (etanercept), (4) concurrent existence of infectious or ischemic colitis and (5) patients with Crohn's disease type L4B2 and L4B3 classified according to Vienna classification system[8].

Crohn's disease was confirmed by findings on barium radiography, endoscopy, and histopathology, as described by others[9]. The activity of Crohn's disease among patients was defined according to Crohn's Disease Activity Index (CDAI). Patients with CDAI score ranging between 150 and 400 were thought to have mild activity of the disease and patients with CDAI score ranging between 400 and 600 were thought to have severe activity. Remission was considered when CDAI score was less than 150[10].

The diagnosis of ulcerative colitis relied on history, stool examination, the sigmoidoscopic or colonoscopic appearance and the histological assessment of rectal or colonic specimens. Activity of ulcerative colitis was defined by its clinical and endoscopic severity. According to its clinical severity patients were categorized as suffering from disease remission, or mild, moderate, and severe disease as described previously; the latter disease activity was graded between 0 and 3, respectively[11]. According to endoscopic findings a system scaling between 0 and 4 was applied. The latter system is as follows: 0: normal mucosa, 1: loss of vascular pattern, 2: granular mucosa, 3: friability on rubbing and 4: spontaneous bleeding or ulceration of the mucosa[12].

Patients with no clinical, laboratory, histological and endoscopic abnormalities who underwent colonoscopy served as controls. The study was conducted in accordance to the Helsinki Declaration. Upon admission, a total of 20 mL of blood were collected after puncture of one forearm vein. Ten mL was added into 40 mL of trypticase soy broth (Becton Dickinson) for quantitative blood culture. The other 10 mL of blood was collected in a sterile tube and centrifuged; the supernatant was kept at -70℃ until assayed. Serum was applied for the estimation of sTREM-1, TNFα and malondialdehyde (MDA).

Estimation of sTREM-1

Estimation of sTREM-1 was performed by a home-made enzyme immunoassay. Capture antibody of sTREM-1 (R&D Inc, Minneapolis, USA) was diluted to 4000 mg/L and distributed in a 96-well plate at a volume of 0.1 mL per well. Samples of serum were centrifuged at 12 000 g for 10 min and the supernatants were removed. After overnight incubation at 25℃, wells were thoroughly washed with a 0.5 g/L solution of Tween in PBS (Merck) (pH: 7.2-7.4). Then 0.1 mL of standard concentrations of sTREM-1 (15.1-4000 ng/L, R&D Inc) diluted with reagent diluent (10 g/L BSA in PBS, pH 7.2-7.4, 0.2 micron filtered) serving as a buffer or of serum was added in wells. After incubation for two hours, wells were washed thrice, and 0.1 mL of one 400 ng/mL dilution of sTREM-1 detection antibody (R&D Inc) was added per well. The plate was then incubated for two hours, and attached antibodies were signalled by streptavidin. Concentrations of sTREM-1 in each well were estimated by the optical density detected at 450 nm after addition of one 1:1 solution of H_2O_2: tetramethylbenzidine as a substrate (R&D Inc). sTREM-1 concentration was expressed in ng/L. All determinations were performed in duplicate; the inter-day variation of the assay was 5.23%.

Estimation of TNFα

Tumor necrosis factor alpha was measured in serum with an enzyme immunoassay (Amersham, London, UK). Lowest limit of detection was 0.5 ng/L. All measurements were performed in duplicate and cytokine concentrations were expressed as ng/L.

Estimation of malondialdehyde (MDA)

Lipid peroxidation was estimated by the concentration of MDA, as already described[13]. Briefly, a 0.1 mL aliquot of each sample was mixed to 0.9 mL of trichloroacetic acid 200 g/L (Merck) and centrifuged at 12 000 g, 4℃ for 10 min. The supernatant was removed and incubated with 2 mL of thiobarbituric acid 2 g/L (Merck) for 60 min at 90℃. After centrifugation, a volume of 10 μL of the supernatant was injected into a high-performance liquid chromatography system (HPLC, Agilent 1100 Series, Waldbronn, Germany) with the following characteristics of elution: Zorbax Eclipse XDB-C18 (4.6 mm × 150 mm, 5 μm) column below 37℃; mobile phase consisting of a 50 mmol/L K_3PO_4 (pH 6.8) buffer and methanol 990 g/L at a 60/40 ratio with a flow rate of 1 mL/min; fluorometric detection with signals of excitation at 515 nm and emission at 535 nm. The retention time of MDA was 3.5 min and it was estimated as μmol/L by a standard curve created with 1, 1, 3, 3-tetramethoxy-propane (Merck). All determinations were performed in duplicate.

Statistical analysis

Comparison between groups was made by Mann-Whitney U test. Correlations between concentrations of sTREM-1, TNFα and MDA and severity indexes were performed according to Spearman's rank of order. $P < 0.05$ was considered as significant.

RESULTS

Patients' clinical and demographic characteristics are shown in Table 1. Blood cultures obtained from patients of all study groups were sterile. Concentrations (Median ± SE) of sTREM-1 of controls, patients with Crohn's disease, and patients with ulcerative colitis were 53.31 ± 32.93, 735.10 ± 197.17 ($P = 0.008$ *vs* controls), and 435.82 ± 279.71 ($P = 0.049$ *vs* controls) ng/L, respectively (Table 1). Respective concentrations of TNFα were 6.02 ± 3.94, 7.98 ± 5.08 (P = NS *vs* controls), and 8.45 ± 4.15 ($P = 0.018$ *vs* controls) ng/L (Table 1). Respective concentrations of MDA were 0.93 ± 0.23, 1.46 ± 0.56, and 1.49 ± 0.61 ($P = 0.041$ *vs* controls) μmol/L (Table 1). The concentrations

Table 1 Demographic data of IBD patients

Demographic data	Control group	Crohn's disease	Ulcerative colitis
Gender (Male/Female)	12/7	3/5	18/13
Age (mean ± SD, yr)	64.2 ± 14.8	29.4 ± 13.0	43.8 ± 16.4
White blood cells (mean ± SD, x 10^9/μL)	7.9 ± 2.3	9.0 ± 3.0[a]	9.5 ± 3.6[a]
Platelets count (mean ± SD, x 10^9/μL)	237 ± 56	187 ± 38[a]	179 ± 33[a]
Blood cultures	sterile	sterile	sterile
Clinical activity index of UC (mean ± SD)	-	-	1.52 ± 0.26
Endoscopic activity index of UC (mean ± SD)	-	-	1.65 ± 0.45
Crohn's disease activity index (mean ± SD)	-	331.81±117.25	-
TNFα (median ± SE, ng/L)	6.0 ± 3.9	8.0 ± 5.1	8.4 ± 4.2[a]
sTREM-1 (median ± SE, ng/L)	53 ± 33	735 ± 197[b]	435 ± 279[a]
MDA (median ± SE, μmol/L)	0.93 ± 0.23	1.46 ± 0.56	1.49 ± 0.61[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* controls.

Table 2 sTREM-1, TNFα and MDA in IBD patients

Activity index	TNFα (ng/L)	sTREM-1 (ng/L)	MDA (μmol/L)
UC Clinical AI			
Grade 0	4.19 ± 1.52	7.09 ± 4.90	1.74 ± 0.11
1	5.13 ± 0.98	18.91 ± 15.00	1.87 ± 1.05
2	4.98 ± 0.42	288.28 ± 133.82[b]	1.83 ± 0.46
3	9.98 ± 1.23[a]	1596.45 ± 397.38[b]	1.97 ± 0.31
UC endoscopic AI			
Grade 0	4.33 ± 1.51	4.91 ± 3.21	1.36 ± 0.11
1	5.13 ± 0.98	18.91 ± 15.00	1.87 ± 1.05
2	4.86 ± 0.41	288.28 ± 133.82[b]	1.66 ± 0.43
3	8.98 ± 0.42[a]	1108.19 ± 309.95[b]	1.74 ± 0.39
4	21.99 ± 10.50[a]	2281.25 ± 556.77[b]	1.91 ± 0.50
CDAI			
Score < 150	4.88 ± 1.44	4.91 ± 3.21	1.78 ± 0.11
150-400	5.21 ± 0.88	16.03 ± 4.19	1.88 ± 0.67
400-600	5.67 ± 0.91	2281.25 ± 556.77[a]	1.95 ± 0.95

[a]$P < 0.05$, [b]$P < 0.01$ *vs* grade 0 and score < 150.

of sTREM-1 were higher in patients with severe degree of Crohn's disease than in patients in remission of Crohn's disease ($P = 0.032$, Table 2).

Correlation between sTREM-1 and TNFα of patients with ulcerative colitis was significant ($P = 0.001$) (Figure 1). No correlation in patients with ulcerative colitis was found between sTREM-1 and MDA, and TNFα and MDA, consecutively; nor was correlation found in patients with Crohn's disease between sTREM-1 and TNFα, sTREM-1 and MDA and TNFα and MDA.

DISCUSSION

Triggering receptor expressed on myeloid cells (TREM-1) is a recently discovered receptor on cell membranes of neutrophils and monocytes involved in inflammatory processes and activated by microbial components. Engagement of TREM-1 has been reported to trigger the synthesis of proinflammatory cytokines[4]. sTREM-1 is the soluble counterpart of TREM-1 that is increased in gastric juice of patients with peptic ulcer disease[7]. Based on the latter involvement of sTREM-1 in diseases of the gastric mucose, it was hypothesized that sTREM-1 might be implicated in inflammation of intestinal mucosa. Our data revealed that sTREM-1 was increased in the sera of patients with ulcerative colitis and Crohn's disease (Tables 1 and 2). Various hypotheses could be made to explain the mechanism of increase of sTREM-1 based on the role of bacteria and fungi to trigger TREM-1[14]. It might be assumed that sTREM-1 was increased as a consequence of two probable conditions related with a microbial infection; (1) bacteremia as a result of bacterial translocation through the inflamed intestinal mucosa, and (2) infectious colitis. Both mechanisms are unfavorable for the following two reasons: (1) patients with infectious colitis were excluded from the study, and (2) quantitative blood cultures obtained from patients of all study groups were found sterile. Furthermore no differences were found for white blood cell and platelet counts between controls and patients with

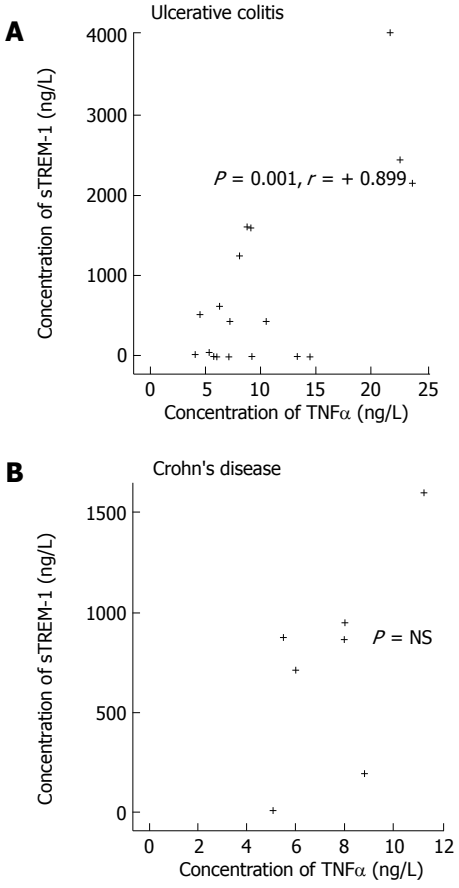


Figure 1 Correlations between sTREM-1, TNFα of IBD patients.

ulcerative colitis as well as between controls and patients with Crohn's disease (Table 2). So, it might be presumed that sTREM-1 increase was not a result of a microbial infection.

The similar kinetics of sTREM-1 and TNFα might indicate the implication of sTREM-1 in the pathogenetic mechanisms leading to IBD. Evolution of IBD seems to

result from the derangement of balance between pro-inflammatory cytokines and mediators with anti-inflammatory activity. The latter imbalance contributes to impairment of mucosal barrier function leading to colonic inflammation[15]. sTREM-1 was increased only in IBD of moderate and severe degree signifying the possibility that sTREM-1 might be an indicator of the severity of the colonic inflammation (Table 2) reflecting the degree of the inflammatory reaction taking place in the intestinal mucosa.

The significance of MDA increase in IBD has already been described by others[16,17]. MDA is a compound functioning as an indicator for the severity of oxidative stress. Oxidative stress seems to be involved in the pathogenesis of several inflammatory processes[16,17]. In the present study, MDA was not correlated with sTREM-1. The latter finding could be explained due to the fact that MDA is an indicator of lipid peroxidation that is not specific to IBD. The present data show for the first time, to our knowledge, that sTREM-1 levels correlate with clinical disease degree and TNFα levels in ulcerative colitis, raising the possibility that it is a mediator in this condition. Further research is mandatory to elucidate the role of sTREM-1 in the evolution from the initial immunologic events occurring in the inflamed intestinal mucosa to the development of inflammatory bowel disease.

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RAPID COMMUNICATION

Prevalence of occult HBV infection in haemodialysis patients with chronic HCV

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Abstract

AIM: To study the prevalence and clinical effects of occult HBV infection in haemodialysis patients with chronic HCV.

METHODS: Fifty chronic hemodialysis patients with negative HbsAg, and positive anti-HCV were included in the study. These patients were divided into two groups: HCV-RNA positive and HCV-RNA negative, based on the results of HCV-RNA PCR. HBV-DNA was studied using the PCR method in both groups.

RESULTS: None of the 22 HCV-RNA positive patients and 28 HCV-RNA negative patients revealed HBV-DNA in serum by PCR method. The average age was 47.2 ± 17.0 in the HCV-RNA positive group and 39.6 ± 15.6 in the HCV-RNA negative group.

CONCLUSION: The prevalence of occult HBV infection is not high in haemodialysis patients with chronic HCV in our region. This result of our study has to be evaluated in consideration of the interaction between HBsAg positivity (8%-10%) and frequency of HBV mutants in our region.

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Key words: Chronic HCV; Haemodialysis; Occult HBV infection

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INTRODUCTION

In most cases, occult HBV infection is defined with low existence of HBV infection in HbsAg deficiency. Serum HBV-DNA level in these patients is generally lower than 10^4 copies/mL. It is reported that there is a high prevalence of occult HBV infection in patients with chronic hepatitis C, HCC and haemodialysis patients, criptogenic liver disease, drug injection users and HIV patients, and in those undergoing frequent blood transfusion (those with hemophilia disease, etc.) and blood donors[1-8]. The existing data is limited on occult HBV among patients on long-term dialysis. It has been recently suggested that occult HBV infection might be associated with a lower response of hepatitis C virus (HCV) to standard interferon therapy in patients with normal renal function. The prevalence of occult HBV infection in renal dialysis patients ranges between 0% and 58% in published reports. The aim of this survey is to address epidemiological and clinical significance of occult HBV infection in patients receiving regular dialysis in Turkey.

MATERIALS AND METHODS

A total of 50 patients with chronic renal failure receiving haemodialysis have been chosen with negative HbsAg and positive anti-HCV for at least 6 months and accepted for this study from among the patients of dialysis unit, and from among patients who have applied to Internal Diseases, Faculty of Medicine, Dicle University. These patients were divided into two groups, HCV-RNA positive (22 patients) and HCV-RNA negative (28 patients). Average haemodialysis periods were 4.9 ± 2.3 years in the HCV-RNA positive group[1-9] and 4.6 ± 2.1 years in the HCV-RNA negative group[1-8]. There were 13 male and 9 female subjects in the HCV-RNA positive group, with an average age of 47.2 ± 17.0 years[18-24]. There were 17 male and 11 female subjects in the HCV-RNA negative group, with an average age of 39.6 ± 15.6 years[13-16]. Cirrhosis existence in patients was externalized both clinically and through laboratory tests.

Fifteen ml blood was taken from the patients, centrifuged at 2500 r/min for 5 min, and stored at -25 ℃ with separated serum samples. Hepatitis panel (HBsAg, anti-HBs, anti-HBc IgG) was studied by "Macro Enzyme Immune Assay (Macro EIA) " method in Elecsy 2010 (Roche) device and with similar brand kits; and anti-HCV was studied with the Cobas Core II Immunochemical system (Roche). The "Real-time PCR method" with Robo Gene

(Roboscreen, Leipzig, Germany) kits in ABI PRISM 5700 (Applied Biosystems) device HCV-RNA was used. HBV-DNAs of cases included in the study were quantitatively evaluated by PCR method. Techne Cylogene Thermal Cycler (Techne Cambridge Ltd) Duxford-Cambridge U.K. device was used for HBV-DNA PCR amplification. For PCR amplification, viral DNA was insulated from 100 μL serum in the incubation tampon containing 1 mg/mL Proteinase K at 37℃ through phenol-chloroform extraction and ethanol precipitation following a 3-h incubation.

RESULTS

Patients included in the study were divided into two groups, HCV-RNA positive and HCV-RNA negative. Demographic and clinical characteristics of these patients are shown in Table 1. Thirty (60%) of 50 patients were male, while 20 (40%) patients were female, with an age range of 13-84 years, and averaging 42.9 ± 16.5 years.

HCV-RNA PCR examination was positive in 22 (44%) patients, and negative in 28 (56%) patients. No HBV-DNA (0%) was detected through the high-sensitivity PCR study in both patient groups with positive and negative HCV-RNA. Accordingly, occult HBV infection was detected in none of the 50 patients in our study.

Results in anti-HCV positive and HCV-RNA positive group (22 patients) are as shown in Table 1. Considering both groups together, the results in 50 patients are as follows.

Serum ALT levels are above normal in 12 (24%) of the 50 patients, 8 showed persistent and meaningful ALT level in their six-month monitoring. HCV-RNA was positive in 7 of the 8 patients, and negative in 1 patient. With regard to the duration of haemodialysis, only 6 of the 50 patients were receiving haemodialysis for less than 3 years, 44 patients were receiving haemodialysis treatment for 3 years and over. The minimum haemodialysis duration was 1 year. Twelve (57.1%) out of a total of 21 patients in the two groups with insulated anti-HBs seropositivity received HBV vaccination in their history.

Table 1 Demographic and clinical characteristics of patients

	HCV-RNA Positive (*n* = 22)	HCV-RNA Negative (*n* = 28)	Total (*n* = 50)
Average age (yr) (age range)	47.2 ± 17 (18 - 24)	39.6 ± 15.6 (13-76)	42.9 ± 16.5 (13 - 84)
Sex Male	13 (59.1%)	17 (60.7%)	30 (60%)
Sex Female	9 (40.9%)	11 (39.3%)	20 (40%)
Average Haemodialysis period (yrs) (haemodialysis range)	4.9 ± 2.3 (1 - 9)	4.6 ± 2.1 (1-8)	4.8 ± 2.2 (1-9)
ALT (IU/mL)	52.9 ± 52.8 (4 - 186)	29.3 ± 20.9 (2-88)	39.7 ± 39.7 (2 - 186)
Insulated anti-HBs Seropositivity	7 (31.8)	14 (50%)	21 (42%)
Insulated anti-HBc IgG seropositivity	3 (13.6%)	1 (3.5%)	4 (8%)
Anti-HBs and anti-HBc IgG Seropositivity	10 (45.4%)	6 (21.4%)	16 (32%)
Anti-HBs and anti-HBc IgG Seronegativity	2 (9.1%)	7 (25%)	9 (18%)
HBV-DNA (PCR) Positivity	0 (0%)	0 (0%)	0 (0%)

DISCUSSION

In most cases, occult HBV infection is defined with low existence of HBV infection in HbsAg deficiency. Serum HBV-DNA level in these patients is generally lower than 10^4 copies/mL. It is reported that there is a high prevalence of occult HBV infection in patients with chronic hepatitis C, HCC and haemodialysis patients, in those with criptogenic liver disease, drug injection users and HIV patients, and in those who underwent frequent blood transfusion (those with hemophilia disease, etc) and blood donors[1-8].

As they are similar transmission models, HBV and HCV co-infection are prevalent clinical presentations. Anti-HBc prevalence reported in patients with chronic HCV infection is 50%-55%[1,2,9]. This ratio is a close value, 59% (13/22) in our study. Most studies show that HBV-DNA genome existence is 22%-87%[10,11] in patients with negative HBsAg and positive HCV-RNA. HBV-DNA is seen in 46% of anti-HBc positives, and in 20% of anti-HBc negatives[11]. In chronic HCV related liver diseases, frequency of detectable HBV-DNA is apparently higher than HCV non-related liver diseases[11]. However, variable prevalence of occult HBV infection is seen in the permanence of HBV-DNA PCR[12]. And this makes us think that HBV viremia shows fluctuations during occult HBV infection.

Clinical interaction of HCV and occult HBV infection is still controversial. Some studies report that cirrhosis is seen more frequently in those patients with HCV and occult HBV co-infection than in those with HCV infection alone[11]. However, there are also studies that are not in support of this[13]. Parallel to this, HCV and occult HBV co-infection had a lower incidence in non-cirrhotic hepatitis[14]. Occult HBV co-infection is related to higher ALT levels and histological activity. Despite this, other studies reveal that the incidence of liver related complication in these patients is at a level comparable to those with HCV infection alone[9-13]. Patients with active HBV and HCV co-infection tend not to respond to interferon treatment. Some studies show that such low interferon response exists in patients with HCV and occult HBV co-infection[14-16]. However, other studies reveal that occult HBV co-infection has no effect on the interferon response of HCV infection. Interferon treatment may accelerate HCV clearance in patients with HCV and occult HBV co-infection, but HBV-DNA remains at detectable level. HCV-RNA level is apparently higher in patients with occult HBV co-infection than patients with HCV infection alone. Despite all these, three recent studies show a lower prevalence of chronic HCV and occult HBV co-infection and that the effects of occult HBV infection on chronic HCV infection remained low in these patients[17-19]. While one of these studies report occult HBV co-infection prevalence at 6.7% in patients

with chronic HCV infection, no difference was found in the frequency of occult HBV infection between those with and without a marker for HBV infection in their history[18]. Another study found that occult HBV infection had no effect on the early phase response of chronic HCV infection combined treatment[17]. And the last study states that occult HBV infection has no significant effect on treatment response upon HCV viral titre, liver enzymes, histological parameters and HCV and interferon-α and ribavirin in patients with chronic hepatitis C[19].

In a study on chronic haemodialysis patients, serum in PCR and HBV-DNA in PBMC was studied in 67 patients. While serum HBsAg and HBV-DNA were found negative in all of these patients, HBV-DNA was positive in 5 patients (7.5%) in PBMC[6].

Another study focused on the disease etiology in 107 patients with negative HCC and cirrhosis HbsAg and 192 chronic hepatitis patients as control group with negative HbsAg. In the group with HCC, HCV was blamed as etiological factor in 73 (68%) patients, criptogenic liver disease in 29 (27%) patients and alcohol in 5 (5%) patients. HCV was blamed in 153 (80%) patients of the group with chronic hepatitis HCV, criptogenic liver disease was blamed in 32 (17%) patients and alcohol was blamed in 7 (4%) patients[2]. HBV-DNA PCR was checked in these patients, being positive in 68 (64%) patients with HCC, 63 (33%) patients with chronic hepatitis. In patients with HBV-DNA positive HCC, anti-HBc positivity was found higher than anti-HBc negativity percentage (64% and 56%, respectively). Frequency of occult HBV infection varies in patients with HCC. The prevalence of anti-HBc/Anti-HBs is 43% in such patients, and the HBV-DNA prevalence varies between 5%-80%[2-20].

Although there are strong evidences aimed at the relationship between occult HBV infection and HCC, the role and place of HBV is not clear in this series of incidents. Is it a silent bystander, a cofactor, or criminal alone? There are strong evidences about the etiological relationship between chronic HBV infection and HCC. It is unlikely that occult HBV infection is only a silent bystander in patients with HCC. The high prevalence of occult HBV infection in patients with HCC supports the role of occult HBV infection in the development of HCC compared with the results in patients with cirrhosis or chronic hepatitis. It is quite possible that occult HBV infection plays a role as cofactor in HCC development. Two viruses may mutually interact, causing more painful inflammation and quicker cirrhosis progress. Alternatively, direct oncogenic effect of two viruses may be additive or synergistic[2]. This has been an issue of interest in patients with occult HBV coinfection. Evidences relating to occult HBV infection in patients with HCC related to HCV are being gradually seen more frequently[1,2,4]. In a study made in Spain, in 109 anti-HCV negative and HBsAg negative patients with increased liver enzymes with unknown etiology, 19% HBV-DNA was detected in serum[21]. Again in another study, in 50 patients with criptogenic chronic hepatitis, there was detectable HBV-DNA in 15/50 (30%) patients. Of these 15 patients, 66% had increased liver enzymes and 53% suffered from painful fibrosis and cirrhosis. In their repeated liver biopsies during follow-up, in 2/11 (18.2%) patients, the disease progressed from chronic hepatitis to cirrhosis. Generally considering, clinical results of occult HBV infection after delayed HBsAg clearance vary. These results depend upon the underlying liver disease, duration of active HBV infection and degree of liver damage before HBsAg clearance[1,2].

An increased frequency of occult HBV infection was reported in immune-dominant patients[1]. Lymphoma, aplastic anemia or cythotoxic treatment for leukemia, allongenic or otolog bone marrow transplantation practice in patients with occult HBV infection chronic might cause reactivation of Hepatitis B or development of fulminant hepatic insufficiency[22,23]. However, chronic hepatitis B reactivation with induced treatment is less frequent and painful in those with occult HBV infection before the treatment than those with chronic hepatitis B with positive HBsAg before the treatment[1]. Viral infection progresses with the application of immune-suppressive agent, destruction starts with immune intermediary in hepatocytes infected with HBV after the regaining of immunity with the interruption of this agent.

In patients with fulminant hepatic insufficiency, the pathogenetic role of occult HBV infection has been very well defined. In these patients, positivity of serum HBV-DNA ranges between 0%-47%[24-26]. Recurrence of HBV infection following liver transplantation for fulminant hepatic insufficiency shows possible HBV replication in these patients[24,25]. However, the pathogenetic role of occult HBV infection in patients with fulminant hepatic insufficiency is doubtful and weak. A multi-centered study in the USA shows that occult HBV infection is not related with acute liver insufficiency[26].

Occult HBV infection was found in 81/180 patients (45%) in drug injection users in Baltimore[7]. In a study, 10% occult HBV infection was detected in patients with HIV[8]. In Japan, occult HBV infection was detected in 22 (51.2%) of 43 hemophilia patients[5]. It has been reported that in numerous studies covering wide populations conducted in many different countries, occult HBV infection incidence varied between 0%-15% in blood donors[3].

Most of the relevant authors are mostly unable to provide clear replies to the question what is the responsibility and effect of occult HBV infection in the pathogenesis of diseases where there is a high prevalence of occult HBV infection and where the pathogenetic role of apparent HBV infection is well known. And they are still suspicious on most things. Although there are questions in minds about the subject, pathogenetic role of occult HBV infection is accepted best in HCC.

It is reported that the prevalence of occult HBV infection is parallel with the prevalence of apparent HBV infection in that region[1,2]. For example, occult HBV infection prevalence varies between 7%-19% among blood donors in endemic regions where 70%-90% of the population are exposed to HBV[2]. In Western countries (e.g., there is pre-reported HBV exposure in 5% of the American population), frequency of occult HBV infection ranged between 0%-9%[2]. Prevalence of occult HBV infection can also be affected by incorrect negative and incorrect positive results based on the sensitivity and specificity of the HBV-DNA PCR method. Therefore, the

specificity of the methods has to be verified and cross-contamination has to be prevented. Moreover, in order to positively consider the result in HBV-DNA PCR method, one needs to show at least two different primers from different areas of the HBV genome clone. Most of the studies so far mentioned the sample collection method at very few occasions[1,2,4]. Cross contamination may occur particularly in retrospective studies where the blood samples used in the past are collected.

In a study made by Beşışık *et al*. in 33 patients with chronic hepatitis C and at the same time chronic haemodialysis with negative HBsAg and positive HCV-RNA, serum HBV-DNA PCR study yielded positive results in 12 (36.4%) patients. YMDD mutation was detected in 6 (50%) of these 12 patients[27]. According to this study, there is increased occult HBV infection incidence in patients with chronic hepatitis C treated with chronic haemodialysis and YMDD mutation is the responsible agent in an important part of them. In our study, according to the HCV-RNA condition of the 50 cases chronic haemodialysis with negative HbsAg and positive anti-HCV, the study group was divided into two groups, positive HCV-RNA (22 patients) and patients with negative HCV-RNA (28 patients). None (0%) of the patient groups revealed HBV-DNA in serum by PCR method despite the HCV-RNA positive group has been studied twice in different times. Contrary to the above-mentioned similar study, there is no increased incidence of occult HBV infection in patients with chronic haemodialysis with chronic hepatitis C in our region. In our study, 3 patients showed insulated anti-HBc IgG seropositivity and positive HCV-RNA (13.6%), and 1 patient showed negative HCV-RNA (3.5%). And the percentage of having at least one of the markers of past HBV infection was 89.9% (20 patients) in HCV-RNA positive group and 75% (21 patients) in HCV-RNA negative group. However, no increase was detected in occult HBV infection incidence in our sturdy.

The result of our study has to be evaluated in consideration of the interaction between the HBsAg positivity (8%-10%) for Diyarbakır region and the frequency of HBV mutants in this region. Besides, it has become necessary to review the sensitivity and specificities of still non-standardized HBV-DNA PCR methods and define a common method of diagnosis accordingly.

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RAPID COMMUNICATION

Expression of cyclooxygenase-2 is associated with p53 accumulation in premalignant and malignant gallbladder lesions

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Abstract

AIM: To examine the relationship between cyclooxygenase-2 (COX-2) overexpression and p53 accumulation in gallbladder carcinoma and its precursor lesions.

METHODS: Sixty-eight gallbladder tissue samples comprising 14 cases of normal gallblader epithelium, 27 cases of dysplasia (11 low-grade dysplasia and 16 high-grade dysplasia) and 27 adenocarcinomas were evaluated by immunohistochemistry for COX-2 expression and p53 accumulation. The relationship among COX-2 expression, p53 accumulation and clinicopathological characteristics was analysed.

RESULTS: COX-2 was expressed in 14.3% of normal gallbladder epithelium, 70.3% of dysplastic epitehlium, and 59.2% of adenocarcinomas. When divided into low- and high-grade dysplasia, COX-2 was positive in 5 (45.4%) cases of low-grade and 14 (87.5%) of high-grade dysplasia (P = 0.019). Accumulation of p53 was detected in 5 (31.2%) cases of high-grade dysplasia and in 13 (48.1%) of carcinomas. No p53 accumulation was found in normal epithelium or low-grade dysplasia. COX-2 overexpression was observed in 17 of 18 (94.4%) cases with p53-accumulation in comparison with 20 (40.0%) out of 50 cases without p53 accumulation (P < 0.001).

CONCLUSION: The significant differences in COX-2 expression among normal epithelium, low-grade dysplasia and high-grade dysplasia suggest that overexpression of COX-2 enzyme is an early event in gallbladder carcinogenensis. Furthermore, since accumulation of p53 correlates with COX-2 expression, COX-2 overexpression observed in gallbladder high-grade dysplasia and carcinoma might be partly due to the dysfunction of p53.

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Key words: Gallbladder; Carcinogenesis; Cyclooxygenase-2; p53; Immunohystochemistry

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INTRODUCTION

Gallbladder carcinoma is the fifth most common malignancy of the digestive tract. Recent molecular genetic studies have shown that selected proto-oncogenes and tumor-suppressor genes are involved in the development and progression of gallbladder carcinoma, and a different spectrum of molecular genetic changes appears to be responsible for each of the different preneoplastic conditions[1]. An association of gallbladder carcinoma with cholelithiasis or an anomalous arrangement of the pancreaticobiliary duct suggests that long-term inflammation may modulate tumorigenesis as well as the progression of this type of carcinoma[2,3]. Based on the histopathological examination of surgically resected gallbladder, an assumption has been made that epithelial changes found adjacent to invasive carcinomas in the gallbladder wall are premalignant and may progress to carcinoma. The rarity of most presumptive premalignant conditions has made it difficult to obtain better knowledge of the biological characteristics of precursor lesions of gallbladder carcinoma. At present, three major putative pathways of gallbladder carcinogenesis seem to be important: dysplasia, adenoma and anomalous pancreaticobiliary ductal junction. Roa[4] suggested that the period required to transform dysplasia into an advanced carcinoma is about 15 years. However, gallbladder carcinomas are usually diagnosed at an advanced stage, representing a challenge in the management because they respond poorly to therapy and are therefore associated

with a poor prognosis. A postoperative 5-year survival rate between 5% and 13% has recently been reported[5,6]. Since this type of cancer is so difficult to cure by surgery alone, new molecular targets are needed for its prevention and treatment.

Prostaglandins (PGs), a family of lipid-derived autocrine and paracrine mediators, can favor tumorigenesis by altering cell proliferation, differentiation and adhesion, such as by modulating vascular response and immune surveillance. PGs are derived from arachidonic acid. The key step in the conversion of free arachidonic acid to PG is catalysed by the cyclooxygenase enzyme (COX)[7]. Three isoforms of COX (COX-1, COX-2 and COX-3) have been identified. While COX-1 is constitutively expressed as a housekeeping gene in most cells, COX-2 is not detected in normal tissues, except in kidney, liver and pancreatic islands, although it is expressed as an early response to many stimuli, such as inflammatory cytokines, growth factors and oncogens[8]. There is only little information about the recently identified COX-3 isoform. PGs have been shown to mediate gallbladder inflammatory response, since non-steroidal anti-inflammatory drugs (NSAIDs) have been proved to decrease gallbladder inflammation[9]. Accumulating evidences suggest that increased PGs levels via overexpression of the inducible COX-2 isoform are important in the development of human cancer[10]. Though increased levels of PGs and COX-2 expression have frequently been found in malignant growths of the digestive tract (colon, stomach, oesophageal cancer) similar data about biliary tumors are rare[11,12].

Point mutations or deletions of the p53 gene are observed in approximately 50% of cancers. Wild-type p53 is a key molecule in the cellular DNA damage response, causing restriction of cell proliferation by inducing cell cycle arrest and apoptosis. The product of mutant p53 has often been found to be a stable protein with a long half-life, while wild-type p53 has a short half-life and is not generally accumulated to a sufficient high level to be detectable by standard immunohistochemistry. An increase in the p53 protein level can therefore be used as an indication of p53 mutation, although there are still some discrepancies[13]. Immunohistochemically, p53 accumulation was detected in 36%-67.8% of gallbladder carcinomas[14-15]. In gallbladder carcinogenesis, an accumulation of p53 protein was reported in association with premalignant transition to malignancy[16]. p53 and COX-2 are thus two important molecules intimately linked to tumorigenesis. COX-2 has been implicated in positive regulation of growth and tumorigenesis, while the tumor suppressor p53 is a negative regulator of these processes. However, there is evidence of COX-2 expression normally suppressed by wild-type p53, suggesting that loss of p53 function may influence COX-2 overexpression[17]. Conversely, COX-2 can in turn inhibit p53-dependent transcription[18].

The purpose of this study was to evaluate the relationship and potential role of COX-2 and p53 in gallbladder carcinogenesis. Immunohistochemically, we analysed the expression profile of COX-2 in gallbladder dysplasia and carcinoma and its correlation with p53 protein accumulation.

MATERIALS AND METHODS

Subjects

A retrospective analysis was performed on 68 gallbladder tissue specimens, including 14 normal epithelium, 11 low-grade dysplasia, 16 high-grade dysplasia and 27 adenocarcinomas, obtained from patients treated with cholecystectomy in the period from 1998 to 2000. No patient had a history of regular ingestion of selected drugs such as NSAIDs or COX-2 inhibitors. We studied 14 cases of normal gallbladder epithelium without dysplastic and/or carcinomatous epithelial changes and 11 with low-grade dysplasia from elective cholecystectomy because of gallstones. In addition, 16 samples of high-grade dysplasia and 27 adenocarcinoma samples were obtained from patients who underwent cholecystectomy due to gallbladder adenocarcinoma. Dysplasia was defined histologically by varying degrees of pseudostratification, nuclear atypias, loss of polarity, and mitotic figures. Depending on the severity of changes, dysplasia was divided into low- and high-grade types. Adenocarcinomas were graded according to the World Health Organisation classification of gallbladder tumors[19]. There were 8 well differentiated, 4 moderately differentiated and 15 poorly differentiated adenocarcinomas.

Methods

COX-2 and p53 expression were investigated by immunohistochemistry. Formalin-fixed, paraffin embedded specimens were sectioned in series at a thickness of 5 μm. After deparafinisation, the slides were immersed for 30 min in 0.3% hydrogen peroxide/methanol to deplete endogenous peroxidase. Antigen retrieval was achieved using the pressure-cooking method for 4 min in citrate buffer (pH = 6.0). For COX-2 expression, polyclonal anti-human COX-2 antibody (Cayman Chemical Company, Ann Arbor, MI, USA) was used in a dilution of 1:500. For p53 analysis, monoclonal mouse anti-human p53 antibody, DO-7 (DAKO, Glostrup, Denmark) diluted at 1:100 was used. After the application of primary antibodies, the specimens were treated with biotinylated secondary antibody for 30 min. Antigen visualisation was achieved by applying a standard streptavidin-biotin complex (ABC, DAKO, Denmark) for 30 min followed by diaminobenzidin chromogen (DAB, Sigma Chemical CO, Germany) in 0.1% H_2O_2 PBS solution. The specimens were counterstained with haematoxylin. The specificity of the applied antibodies was checked with positive or negative controls. Specimens treated without primary antibodies served as negative controls. For positive controls of p53, specimens of human colon carcinoma with known p53 immunoreactivity were processed in the same way as the gallbladder samples. Vascular endothelial cells and fibroblasts observed in all gallbladder specimens provided internal positive controls for COX-2 staining.

Immunostaining was independently evaluated by two investigators (AC and ML) and a consensus agreement was achieved. COX-2 expression was evaluated according to the percentage of positive cells and the intensity of staining. The percentage of positive cells was scored as 0

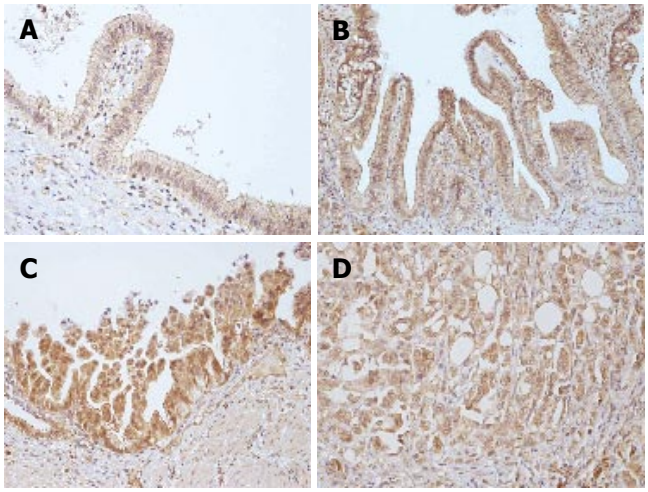


Figure 1 COX-2 expression. **A:** Normal gallbladder epithelium; **B:** Low-grade dysplasia; **C:** High-grade dysplasia; **D:** Gallbladder adenocarcinoma.

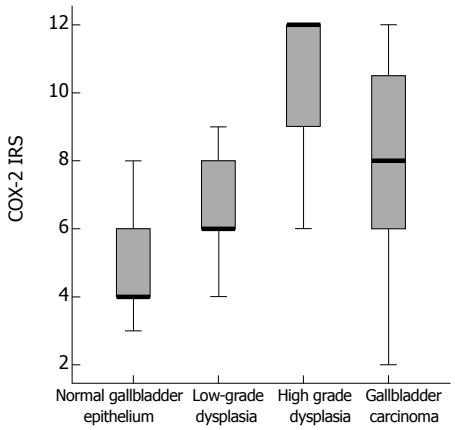


Figure 2 COX-2 – IRS index.

(0%), 1 (< 10%), 2 (10-50%), 3 (51%-80%) or 4 (> 80%). The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). For immunoreactive score (IRS), the percentage of positive cells and the staining intensity were multiplied, resulting in a value between 0 and 12. To separate tumors with weak from those with strong COX-2 expression and to define a cut-off point that might be reproducible for future studies, we united samples with an IRS of 0-6 into one group with negative to weak COX-2 expression (the COX-2 negative group) and those with an IRS of 7-12 (the COX-2 positive group). The minimum requirement for positive IRS was therefore either moderate expression in > 80% of cells or strong expression in > 50% of cells. The expression of p53 was evaluated according to the percentage of positive cell nuclei. The reaction to p53 was considered positive when at least 20% of the nuclei in tumor cells were stained.

Statistical analysis

SPSS 11.0 for Windows (SPSS Inc., Chicago IL) was used. Chi-square tests were used to analyse the correlation between COX-2 overexpression and p53 accumulation and pathological features. The relationship between COX-2 and p53 expression was evaluated by the Mann-Whitney U-test. $P < 0.05$ was considered statistically significant.

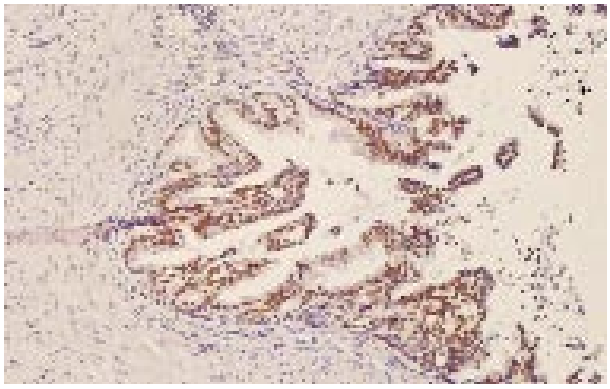


Figure 3 Accumulation of p53 in nuclei of high-grade gallbladder dysplasia.

RESULTS

Surgical specimens of the gallbladder were obtained from 68 patients who had undergone cholecystectomy for clinical chronic cholecystitis or cholelithiasis (n = 51) and primary gallbladder carcinoma (n = 27). There were 43 female and 25 male patients, the median age of patients was 59 years (range from 36 to 78 years). The expression of COX-2 and p53 was analysed by immnohistochemistry in 14 cases of normal gallbladder epithelium, 27 cases of dysplasia and 27 adenocarcinomas. The cytoplasm of gallbladder epithelial cells was stained for COX-2 to various degrees (Figure 1). Additionally, COX-2 immunoreactivity was observed in fibroblasts, endothelial cells, and smooth muscle cells in all specimens. Normal gallbladder epithelium was COX-2 positive (according to the cut-off criteria of the IRS index) in only 2 (14.3%) cases. Of 27 cases of dysplasia, 19 (70.3 %) were COX-2 positive. When we divided this group into low- and high-grade dysplasia, COX-2 was positive in 5 (45.4%) cases of low-grade dysplasia and 14 (87.5%) cases of high-grade dysplasia (P = 0.019). The differences between normal epithelium and low-grade dysplasia were not statistically significant (P = 0.085). COX-2 was positive in 16 (59.2%) of 27 carcinomas.

The greatest positivity for COX-2 was in poorly differentiated carcinomas (73.3%). The differences between high-grade dysplasia and carcinoma were not statistically significant (P = 0.113). COX-2-IRS values detected in normal gallbladder epithelium, dysplasia and carcinoma are shown in Figure 2.

p53 was negative in normal gallbladder epithelium and low-grade dysplasia,. Accumulation of p53 protein was identified in the nuclei of 5 (31.2%) cases of high-grade dysplasia (Figure 3) and 13 (48.1%) carcinomas. Accumulation of p53 protein was the highest (80%) in poorly differentiated gallbladder carcinoma. In the whole spectrum of 68 tissue samples, we found only one COX-2 negative case among 18 p53 positive cases. Of 50 p53 negative cases, 30 (60 %) cases were COX-2 negative ($P < 0.01$).

DISCUSSION

Our analysis of COX-2 and p53 expression in normal, dysplastic (premalignant) and malignant gallbladder epithelium is an attempt to reveal a possible relationship between the two molecules, as well as their role in

gallbladder carcinogenesis. The role of COX enzymes in the process of carcinogenesis has been dramatically emphasized by confirmation that NSAIDs decrease the number of colonic polyps in humans with adenomatous polyposis and lower the incidence of colorectal cancer[20]. Gallbladder cancer has several analogous characteristics with this type of neoplasia. It is preceeded by a lengthy precancerous process taking several years. This process is characterized by chronic inflammation, lithyasis, intestinal metaplasia, and dysplasia in the epithelium of the surrounding mucosa[4]. We observed overexpression of COX-2 in 14 (87.5%) of 16 gallbladder high-grade dysplastic tissues in comparison to 5 out of 11 (45.4%) low-grade dysplasia and 2 (14.3%) of 14 normal gallbladder epithelium. Some slight COX-2 immunoreactivity in normal gallbladder epithelium was not unexpected and could be explained by the well-established connection between COX-2 expression and inflammation[20]. In carcinomas, COX-2 overexpression was found in 16 (59.2%) of 27 gallbladder samples. Our results are consistent with studies by Asano *et al*[11] and Zhi *et al*[21], who found 67.3% and 71.9% gallbladder tumour samples with COX-2 overexpression, respectively. Recently, Tsuchida[3] reported that COX-2 inhibitor meloxicam suppresses carcinogenesis in the gallbladder in an animal model. Our observation that COX-2 overexpression was highest in high-grade dysplasia supports the hypothesis of the role of COX-2 in early carcinogenesis. Liang[22] first suggested that COX-2 expression might be involved in the early proliferative phase but not in the late metastatic phase of the evolution of colorectal cancer. Our study is in agreement with Luzar[23] , who distinguished three histological degrees of gallbladder epithelial changes according to the number of hTERT signals: a) normal and regenerative gallbladder mucosa, b) low-grade dysplasia and c) high-grade dysplasia and adenocarcinoma of the gallbladder. We found no statistical differences in our study in COX-2 expression between normal gallbladder epithelium and low-grade dysplasia ($P = 0.085$), probably due to COX-2 expressed in normal mucosa on account of inflammation. However, the differences were significant between low-grade and high-grade dysplasia. No direct data are yet available about the progression rate of high-grade dysplasia to invasive carcinoma of the gallbladder. For comparison, high-grade dysplasia in the stomach was detected to regress in only 5% of cases, persisted in 14%, and progressed in 81%-85%[24] .

In high-grade dysplasia, p53 protein accumulation was found in 5 (31.2%) cases. Low-grade dysplastic and normal epithelium was p53 negative. Thirteen (48.1%) of 27 carcinomas were p53 positive. The clone against which the p53 antibody was raised (DO-7) results from the recognition of wild type and mutated protein. However, p53 detection was not expected in normal cells. The appearance of a nuclear accumulation of p53 protein might arise from mutation of the protein itself, resulting in failure of its degradation pathway or a mutation of the human mouse double minute (hMDM)-ubiquitin pathway, resulting from the failure of degradation. Accumulation of the wild-type p53 protein owing to a failure of degradation might push the cell into cell cycle arrest and apoptosis. Such cells would occur rather sparsely in tumors because they could not proliferate as malignant clones. It therefore seems very likely that the accumulation of p53 in dysplastic and malignant gallbladder epithelium resulted from p53 mutation[25]. Our results are consistent with some previous reports about p53 gene mutations involved in the carcinogenesis of the gallbladder epithelium[26-27] that p53 overexpression appeard in only 84.6% of gallbladder carcinomas and in 18.7% of patients without carcinoma. Matsubara[28] detected p53 gene mutation in 38.5% of noncancerous biliary epithelial lesions and in 57.1% of adenocarcinomas.

Data about the inter-relationship between p53 and COX-2 are conflicting. Our results are consistent with studies of Shigemasa[29] and Erkinheimo[30]. In both their studies, performed on ovarian carcinoma, a significant positive correlation was found between COX-2 expression and p53 accumulation. The same correlation was accepted in a study of head and neck tumors[31], suggesting that restoration of wild-type p53 expression might interfere with tumour growth by inhibiting the COX-2 pathway. In endometrial carcinoma[32] and colon carcinoma[22], higher COX-2 expression was also associated with p53 accumulation. In contrast, Cho[33] observed no association between COX-2 overexpression and p53 accumulation in renal cell carcinoma. Subbaramaiah[34] demonstrated that wild-type p53 suppressed COX-2 expression by inhibiting its promotor activity. The level of COX-2 protein and mRNA was found markedly decreased in cells expressing wild-type, but not mutant p53[35]. A study of head and neck carcinoma cell lines[36] confirmed that wild-type p53 overexpression has some anti-tumor effects through the suppression of COX-2 gene activity. However, Swamy[18] reported that COX-2 at least in part contributes to the dysfunction of p53 and that celecoxib, a COX-2 selective inhibitor, protects p53 functional activity.

In conclusion, our study is the first to analyse the relationship of COX-2 expression and p53 accumulation in various histological stages of gallbladder epithelial abnormalities and gallbladder adenocarcinoma. Our results suggest that COX-2 might play an important role in gallbladder carcinogenesis. Overexpression of COX-2 appears to be an early event in gallbladder carcinogenesis, already occurring at the stage of preneoplastic epithelial changes (e.g. high-grade dysplasia) and may be, at least in part, related to p53 dysfunction.

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RAPID COMMUNICATION

Glycemic index of cereals and tubers produced in China

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Abstract

AIM: To determine the GI of some cereals and tubers produced in China in an effort to establish the database of glycemic index (GI) of Chinese food.

METHODS: Food containing 50 g carbohydrate was consumed by 8-12 healthy adults after they have been fasted for 10 h and blood glucose was monitored for 2 h. Glucose was used as reference food. GI of food was calculated according to a standard method.

RESULTS: GI of 9 types of sugar and 60 kinds of food were determined.

CONCLUSION: Food GI is mainly determined by nature of carbohydrate and procession. Most of cereals and tubers produced in China have similar GI with their counterparts produced in other countries.

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Key words: Glycemic Index; Cereals; Tubers; Carbohydrate

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INTRODUCTION

GI is a useful index initially coined by Jenkins[1] *et al* to describe the extent to which certain food can increase the blood glucose in human. It is an accurate determination of change of blood glucose after consumption, digestion and absorption of certain food. GIs of 60 kinds of food were determined and reported by Jenkins in 1981. Since then, GI of local foods have been reported by Miller and other scientists[2,3]. Up to date, GI of about 2000 foods have been determined and reported around the world.

The study of GI was limited to clinic research and dietary treatment of diabetes in the early stage of GI study. Since the late 1990s, more and more attention has been paid to the application of GI in body weight maintenance and chronic disease controlling and positive links have been established between diet GI and some pathological conditions such as obesity[4], heart disease[5], diabetes[6].

China is an Asian country with more than thirteen billion people of 56 nationalities and various eating habits and foods. With the rapid development of economy, lifestyle of Chinese people is changing also and a cluster of chronic diseases are emerging, some of which have become health threat of the people as seen in the western countries. Therefore, links between dietary habits and chronic diseases should be investigated and strategies should be developed for the prevention, as well as treatment of such diseases. It is urgent and necessary for us to determine the GI values of Chinese foods. The current study was designed to determine the GI of about 150 types of cereals and tubers by a standard method.

MATERIALS AND METHODS

Subjects

Healthy volunteers (aged 20-45 years, BMI in kg/m^2 range 18.5-25) from Beijing (north China), Chengdu (southwest China), Liaoyang (northeast China) and Yinchuan (northwest China) were involved in the study. All subjects were free from obesity, diabetes and other metabolic diseases and postmenopausal women were excluded. All subjects gave their written informed consent and ethical approval for the study was obtained from the Ethical Committee of Chinese Centers for Disease Control and Prevention.

Test foods: All the foods used in this study were purchased from local supermarket and raw foods were cooked by traditional method used in daily-life of

Chinese. The carbohydrate (CHO) content of the test food was calculated based on food composition data of China according to the following formula: weight of test food = 50/CHO proportion of test food. In cases when CHO content of test food was less than 10%, food weight used to determine its GI was reduced to 25 g. Each food was tested in 10-12 volunteers.

Methods

Oral glucose tolerance test (OGTT): About 10-12 volunteers received OGTT each time. After fasting for 10 h, volunteers were required to arrive at the laboratory at 8 o'clock in the morning. Venous blood samples were obtained before and 15, 30, 45, 60, 90, 120 min after consumption of 50 g glucose dissolved in 200 mL drinking water. Blood was collected into a syringe with sodium fluoride.

Test of glucose response of food: Volunteers with normal glucose tolerance were allowed to receive the glucose response test two days later. When volunteers fasted for 10 h arrived at the laboratory, venous blood samples were collected before and 15, 30, 45, 60, 90, 120 min after consumption of food containing 50 g carbohydrate. For each sample, blood glucose was determined. Each test was repeated and each group of volunteers received no more than tests of three foods. Between tests, at least two days was required for wash-out.

Determination of blood glucose: Calculation of GI: Glucose (GI = 100) was used as reference. The calculation was based on the method developed by Wolever[7]: GI = [(area under the 2-h blood glucose curve for food)/(area under the 2-h blood glucose curve for the same amount of glucose)]× 100

Statistical analysis

The values of GI were expressed as mean ± SD. Dixon test was used to evaluate differences between values of the same group. $P = 0.05$ was selected as level of significance.

RESULTS

Nine sugars were selected as test foods. When glucose was used as the reference (GI of glucose: 100), the GI rank of these sugars from high to low was maltose > white sugar > honey > sucrose > lactose > fructose (Table 1). The GI values of maltose of different source were not equal, which maybe was resulted from procession and the difference of purity.

GI of cereals

Cereals refer to a variety of foods rich in carbohydrate, including traditional starchy food such as steamed bread and noodle, and food processed by modern technology such as breakfast cereals and some instant foods. The significant difference of GI of different cereal foods suggested that food procession and food type were factors influencing the value of GI (Table 2).

GI of some tubers

Tubers also include various types of foods, of which potato, yam, taro and lotus are commonly eaten by Chinese people. Their GI values are shown in Table 3.

Table 1 Glycemic index of some sugars (mean ± SD)

Name	*n*	GI
Glucose	10	65.0 ± 6.3
Fructose	10	23.0 ± 4.6
Lactose	10	46.0 ± 3.2
Chocolate	10	49.0 ± 8.0
Maltose	10	105.0 ± 5.7
Maltose 2	10	75.0 ± 20.3
White sugar	10	83.8 ± 12.1
Honey	10	73.5 ± 13.3

DISCUSSION

Carbohydrate is a major source of energy of human being. Nutrition surveillance in China shows that carbohydrate intake is 360 g/d, which provides 60%-70% of total energy of a person. Cereals and food produced from cereals account for 90% of total dietary carbohydrate. It is obvious that cereals are critical for the health of Chinese.

Because people have realized that overtake of fat is associated with a cluster of chronic diseases, increasing the intake of carbohydrate has been the major concern of nutritionists in dietary recommendation. But controversies still remain as to how and what kind of carbohydrate should be increased in diet.

Food GI is a quantitative physiological parameter used to describe the relative glycemic effect of carbohydrate-containing food compared with reference food (glucose or white bread)[8]. After ingestion, all the digestible carbohydrates are conversed to monosaccharide and absorbed in the intestine, then blood glucose is increased and satiety is ensured. Insulin and other hormones secretion is induced by the increased blood glucose and blood glucose level is then reduced toward the basic level, thus homeostasis is maintained. Avoiding dramatic fluctuation of blood glucose is critical to both the healthy and the diabetic persons. A lower postprandial blood glucose peak and slower decreasing rate are seen after intake of low GI food compared with high GI food. It is evident that carbohydrate content of one food does not always reflect its availability. GI provides a new method for the nutritional evaluation of food.

Food is categorized into three groups according to their GI[8], food with GI < 55 is called low GI food, and high GI food means food with GI >75, food with GI ranging between 55-75 is called medium GI food. Consumption of low GI food will ensure a longer lasting satiety and keep more stable blood glucose, at the same time overtake can be avoided easily compared with consumption of high GI food. High GI food is not good for diabetes and people with impaired glucose tolerance.

GI is a concept comprehensively reflecting the digestibility and utility of food. Factors affecting the GI of food such as composition, content, and type of carbohydrate, physical character and procession are all taken into account in this concept[9,10]. Our results showed that different types of carbohydrate have different

Table 2 Glycemic index of cereal products (mean ± SD)

Food name	*n*	GI	Food name	*n*	GI
Cooked rice	12	83.2 ± 3.1	Steamed bread (refined)	9	88.1 ± 20.2
Brown rice (cooked)	10	87.0 ± 5.0	Wheat pancake	10	79.6 ± 11.5
Sticky rice (cooked)	10	87.0 ± 7.0	Wheat dough, deep-fried	8	74.9 ± 21.0
Sticky rice2 (cooked)	9	88.0 ± 5.6	Bread (refined wheat)	10	87.9 ± 10.2
Sticky rice (higher amylose)	10	50.0 ± 6.0	Bread (whole wheat)	10	69.0 ± 10.4
Rice porridge	10	69.4 ± 18.5	Bread (whole wheat with dried fruit)	10	47.0 ± 7.0
Sticky rice porridge	9	65.3 ± 20.6	Wheat Noodle(dried)	10	46.0 ± 5.8
Black rice porridge	9	42.3 ± 9.0	Wheat Noodle(fresh)	8	81.6 ± 19.1
Rice bran porridge	9	19.0 ± 3.0	Dumpling (shallot + meat)	10	28.0 ± 9.9
			Steamed stuffed bun (shallot + meat)	10	39.1 ± 13.0
Rice cake	8	82.0 ± 7.2	Biscuit (thin)	9	81.0 ± 7.5
Instant rice (in hot water 3 min)	10	46.0 ± 8.5	Biscuit	9	72.0 ± 15.5
Instant rice (cooked 6 min)	10	87.0 ± 5.5	Danone biscuit 1	9	47.1 ± 12.4
Corn powder porridge	10	68.0 ± 10.6	Danone biscuit 2	9	39.3 ± 11.8
Corn granule	10	51.8 ± 9.2	Cake crisp	10	59.0 ± 6.0
Corn flake	9	78.5 ± 12.2	Powder (buckwheat)	8	54.0 ± 3.0
Corn flake 2	9	74.0 ± 10.0	Noodle (buckwheat)	9	59.3 ± 3.0
Oatmeal (unpackaged)	9	55.0 ± 6.1	Bread (buckwheat)	8	66.7 ± 6.0
Oatmeal	8	83.0 ± 18.7	Instant noodle (buckwheat)	8	53.2 ± 5.0
Porridge (instant)	8	69.4 ± 7.2	Millet porridge	8	61.5 ± 9.0
Popcorn	6	55.0 ± 2.0	Powder (various cereals)	9	57.9 ± 3.5
Sweet corn (cooked)	10	55.0 ± 5.0	Whole wheat powder	10	42.0 ± 7.5
Oat biscuit	10	55.0 ± 2.5	Nutritional pancake	8	65.7 ± 5.3
Barley flake	7	69.0 ± 7.3	Biscuit	7	70.0 ± 8.3
Rice (S+R) porridge	9	65.5 ± 16.0	WoTao (Corn+wheat)	10	64.9 ± 16.5

influence on blood glucose. For example, rice rich in amylopectin has high absorption rate and consequently high blood glucose because digestive enzyme can easily reach the structure of starch chain. The opposite is true as to the amylose. Possible explanation for GI difference of different food is: first, food rich in dietary fiber, resistant starch or other indigestible carbohydrate has stronger resistance to digestive enzymes and its digestion and absorption in the intestine is slow and incomplete. Under this kind of conditions, low blood glucose is seen. Second, physical properties of food such as the size of granule and maturity are factors determining GI. Third, time and temperature in procession have significant effect on food GI as seen in the case of potato and vermicelli. It is also obvious that procession is a crucial factor in determining food GI as demonstrated by GI values of different corn, rice, potato and wheat products.

Table 3 Glycemic index of tubers (mean ± SD)

Food	*n*	GI
Potato (cooked)	10	66.4 ± 3.8
Potato (steam)	10	62.0 ± 5.7
Potato crisp (oil fry)	10	60.3 ± 7.0
Potato noodle (with meat)	9	16.7 ± 10.4
Cake (Cushawand potato)	8	108.0 ± 13.4
Yam (steam)	10	51.0 ± 12.0
Yam (cooked)	10	54.0 ± 5.5
Potato mashed	10	73.0 ± 9.2
Potato noodle	9	13.6 ± 2.1
Sweet potato (red, cooked)	8	76.7 ± 12.3
Taro (cooked)	7	47.7 ± 12.7
Pill potato+yam powder	9	34.5 ± 11.7
Lotus root powder	8	32.6 ± 17.0

In accordance with literatures, we found that GI of sucrose is not so high as and the GI of starch is not so low as predicted. The GI of sucrose is lower than expected because sucrose is disaccharide consisting of glucose and fructose which is absorbed and directly transferred to the liver where it is ultimately transformed into glucose and therefore has a lower GI (GI = 23). About half of all the sucrose we ingest is glucose, and fructose accounts for another half. Refined sugar is used in most of processed foods which has a GI of about 60, which is the mean of glucose GI and fructose GI. The GI of starchy foods depends on the rate at which the food can be absorbed and transferred into the blood, and the extent to which the food can raise the concentration of blood glucose. The GI of food with rapid digestibility is usually high. Compared with GI data of other countries, we found that GI of foods produced in China has similar GI to that of counterpart foods produced abroad with the exception of maltose, honey and white sugar. In the present study we found that maltose from different source shows different GI (105 *vs* 75); the GI of honey (67) and white sugar (83) are higher than that published by other authors (GI of honey and white sugar were 58 and 70, respectively). The explanation for this discrepancy is the difference of procession and origin of food. For example, maybe honey and white sugar were supplemented with glucose syrup.

Food of low GI often implies lower utilizable energy. In practice, control of quantity of total energy remains a primary concern in dietary prevention and treatment of obesitic diabetes. GI has been proved a useful and simple concept in food selection, diets arrangement and dietary regulation of blood glucose. GI is not only useful in the dietary treatment of diabetes, hypertension patients and obesity, but also can be applied to dietary

management of athletes, food procession and study of relationship between dietary intake and chronic disease[11]. Establishment of GI values of Chinese foods is the first step of GI study in China. More studies should be conducted in this field.

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RAPID COMMUNICATION

A randomized controlled clinical trial: Interruption of intrauterine transmission of hepatitis B virus infection with HBIG

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Abstract

AIM: To evaluate the efficacy of interruption of intrauterine infection of HBV with HBIG in pregnant women with positive HBeAg and HBsAg.

METHODS: A prospective randomized controlled trial was adopted. Sixty cases with positive HBeAg and HBsAg were coincident with the criteria of inclusion, and 8 cases were excluded. Fifty-two cases were analyzed (28 cases in trial group and 24 in control group). All cases in trial group received 200 IU HBIG intravenously every 4 wk for 3 times from the 28th wk. The cases of control group received placebo in the same way. All pregnant women were detected for HBeAg and HBV-DNA at the beginning of the trial and end of the trial (delivery). The cord blood of all newborns were collected for detecting HBeAg and HBV-DNA simultaneously.

RESULTS: For investigation of HBeAg of newborns in trial group, 6 of 28 cases of newborns had positive HBeAg, the HBeAg positive rate being 21.4%, the total rate of 95% CI being 8%-41%. In control group, 19 of 24 cases of newborns had positive HBeAg, HBeAg positive rate was 79.2%, the rate of 95%CI being 5%-93%. By statistical analysis, χ^2 = 17.26, P < 0.01, RR = 0.27, 95% CI (6.3 × 10^{-6}, 8.6 × 10^{-5}). For investigation of HBV-DNA of newborns in trial group, 7 of 28 cases of newborns had positive HBV-DNA, HBV-DNA positive rate being 25%, the total rate of 95% CI being 11%-45%. In control group, 20 of 24 cases of newborns had positive HBV-DNA, HBV-DNA positive rate was 83.3%, the total rate of 95% CI being 63%-95%. By statistical analysis, χ^2 = 17.62, P < 0.01, RR = 0.30, 95% CI (1.5 × 10^{-5}, 1.7 × 10^{-4}). The results indicated that there was significant difference in HBeAg positive rate and HBV-DNA positive rate of newborns between the two groups. In trial group, 7 of 28 newborns had HBV-DNA positive, but the HBV-DNA load of newborns was lower than that of their mothers. In control group, 20 of 24 newborns still had HBV-DNA positive, and the HBV-DNA load of newborns was close to those of their mothers. Statistical analysis indicated that there was no significant difference in HBV-DNA load between postnatal women without HBIG intervention and their filial generations (T = 81.5, P > 0.1).

CONCLUSION: It is effective and safe to prevent intrauterine infection of HBV with HBIG from the 28th wk in pregnant women with positive HBeAg and HBsAg. In clinical application, those pregnant women with negative HBeAg and positive HBV-DNA also need to be interrupted by HBIG.

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Key words: Interruption; Intrauterine; Transmission; Hepatitis B virus; HBIG

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INTRODUCTION

Hepatitis B virus(HBV) is highly endemic in China. It has been estimated that there are more than 1.2 million of chronic carriers of HBV nationwide. Most of them are infected by mother-infant transmission. Mother-infant infection of HBV can result in chronic HBV carrier state, chronic hepatitis, cirrhosis, hepatecellular carcinoma and the cycling of mother-infant transmission. So to prevent mother-infant transmission is a key strategy to control hepatitis B prevalence in our country. For a long time, it has been successful to vaccinate the infants of HBV positive mother with hepatitis B vaccine or both with hepatitis B immune globulin (HBIG) after birth. However, 10%-20% of the infants have no response to vaccination[1]. The main reason of failing to immunization is intrauterine

infection of HBV. Therefore, it will be meaningful to interrupt the intrauterine infection of HBV. In this study, we try to explore the effects of interruption of intrauterine infection of HBV with HBIG in pregnant women with positive HBeAg. It will be reported as follows.

MATERIALS AND METHODS

Design of clinical trial

A prospective randomized controlled trial was adopted. Trial group and control group were determined by randomized number table. The study (from January. 1997 to May, 2002) were undertaken by 4 sub-centres, including the First Affiliated Hospital, Xinjiang Medical University, Urumqi Military General Hospital; Maternal and Children' s hospital; Xinjiang Jian Gong Hospital. According to the estimation of sample number, 30 cases were included in each group. With regard to the missing of follow-up, we started the clinical trial based on 40 cases in each group.

Subjects

The criteria for inclusion and exclusion were determined before starting the trial. The criteria for inclusion: the pregnant women given pre-labor checkups at obstetric clinic of above hospitals, with positive HBeAg and better general condition, without threatened abortion or threatened premature labor, and hypertension, with normal liver function, and to deliver at the same hospital. The criteria for exclusion: to stop pregnancy for some reasons, to deliver at other hospitals and lose follow-up, and to be administered HBIG not according the regulation.

Sixty cases of pregnant women were coincident with the criteria for inclusion, but 8 cases were excluded, including , 3 cases who stopped pregnancy; 2 cases who transferred to other hospitals; 2 cases who lost to follow-up for unknown reasons and 1 case who used HBIG not according the regulation. So at last, 52 cases were analyzed, including 28 cases in trial group and 24 cases in control group. There was no significant difference about pregnant age, parity, fetal age, delivery mode and median of HBV-DNA (respectively 4.3×10^8 and 3.98×10^8 copy/mL) between those two groups. There was no history of fetal distress in all newborns of the two groups. Fifty-two pregnant women with positive HBeAg labored 52 newborns. No side effects were observed in trial group.

Methods of treatment

All cases in trial group received 200 IU HBIG intravenously every 4 wk for 3 times (28 wk, 32 wk, 36 wk). And side effects were observed. The cases of control group were followed up for checkups, without any treatment.

HBeAg and HBV-DNA test

All pregnant women were detected for HBeAg and HBV-DNA at the beginning of the trial and the labor day. The cord blood of all newborns were collected, separated for serum and stored at -20℃ for detecting HBeAg and HBV-DNA simultaneously. HBeAg was tested by ELISA (commercial kit from Huamei Biotechnology Company, No 20020605), HBV-DNA was tested by FQ-PCR (commercial kit from Da`an Biotechnology Company, No.s19990003). The procedures of detection were performed according to the instruction of the manufacturers.

Table 1 Comparision of cord blood: HBeAg and HBV-DNA positive rate

Groups	cases	HBeAg(+)		HBV- DNA(+)	
	n	*n*	%	*n*	%
Trial	28	6	21.4	7	25
Control	24	19	79.2	20	83.3

Table 2 HBV-DNA levels in 7 mother-infant pairs in trial group (gene copy/mL)

Pair No.	Pregnant women	Filial generation
1	1.16×10^8	1.88×10^5
2	4.45×10^8	2.84×10^4
3	2.74×10^8	1.83×10^5
4	4.78×10^7	3.56×10^5
5	1.53×10^8	2.66×10^5
6	3.94×10^8	2.92×10^4
7	2.28×10^8	1.68×10^5

Statistical analysis

Relative risk (RR) and 95% CI were calculated, χ^2 test was used to compare the difference of qualitative data, Wilcoxon matched rank test was used to compare the difference of HBV-DNA load.

RESULTS

In trial group, 6 of 28 cases of newborns had positive HBeAg (Table 1). HBeAg positive rate was 21.4%, the total rate of 95% CI being 8% and 41%. In control group, 19 of 24 cases of newborns with HBeAg positive (Table 1), HBeAg positive rate was 79.2%, the rate of 95%CI being 5% and 93%. By statistical analysis, $\chi^2 = 17.26$, $P < 0.01$, RR = 0.27, 95% CI: 6.3×10^{-6} and 8.6×10^{-5}. The results indicated that there was significant difference in HBeAg positive rate of newborns between the two groups. The risk of intrauterine transmission could be reduced by immunoprophylaxis with HBIG. In trial group, 7 of 28 cases of newborns had positive HBV-DNA (Table 1), HBV-DNA positive rate was 25%, the total rate of 95% CI being (11%, 45%), In control group, 20 of 24 cases of newborns had positive HBV-DNA, (Table 1), HBV-DNA positive rate was 83.3%, the total rate of 95% CI being 63% and 95%. By statistical analysis, $\chi^2 = 17.62$, $P < 0.01$, RR = 0.30, 95% CI 1.5×10^{-5} and 1.7×10^{-4}. It indicated that there was significant difference in HBV-DNA positive rate of newborns between the two groups. It was effective to decrease the HBV-DNA load of pregnant women with HBV-DNA positive by application of HBIG.

In 28 cases of postnatal women with HBIG intervention, 21 cases of newborns had negative HBV-DNA, 7 cases had HBV-DNA positive, but the HBV-DNA

load was lower than that of their mothers (Table 2). By Wilcoxon matched rank test (T = 28, $P = 0.02$), it indicated that the HBV-DNA levels of filial generation decreased after intervention with HBIG. In control group, only 4 of 24 cases with negative HBV-DNA, the others were still HBV-DNA positive, and the HBV-DNA load was close to those of their mothers. By Wilcoxcon matched rank test (T = 81.5, $P > 0.1$), it indicated that there was no significant difference in HBV-DNA load between postnatal women without HBIG intervention and their filial generations.

DISCUSSION

The prevention of HBV infection by vaccinating newborns has been previously demonstrated to be extremely efficacious. However, some newborns fail to respond to immunization[2]. Intrauterine infection is regarded as the main reason of failure. It has been reported that the rate of intrauterine infection is up to 10%-44%[3]. It shows that intrauterine infection plays an important role in mother-infant transmission of HBV. The intrauterine infection may occur as early as on the 19th wk of pregnancy, but the main time is possibly in the third-trimester of pregnancy[4]. Recently more and more researchers are trying to interrupt the intrauterine infection by intervention. HBIG is a common method to prevent HBV transmission from mother to baby[5,6].

The mechanism of intrauterine infection of HBV has been studied by some researchers[7,8], the findings proved that the main route of HBV transmission from mother to fetus is transplacental. HBIG is an antibody of IgG type. The nourish cells with Fc recepter in placenta can transmit the IgG antibody from mother to infant since the 20 wk during pregnancy. In one study[9], HBIG was injected frequently to pregnant women with positive HBeAg starting from the 20th wk during pregnancy, the positive rate of anti-HBs of newborns was up to 91.42% and no side effect and birth defect were observed in both mothers and newborns. The result showed that anti-HBs can get into the body of infants via placenta after using HBIG during pregnancy. That is to say the mechanism of preventing intrauterine infection of HBV by HBIG is production of fetal passive immunity[9].

This study is a randomized controlled trial to interrupt intrauterine transmission of HBV by HBIG in pregnant women with positive HBeAg. The results showed that it was effective to waken the effectiveness of mother-infant transmission of HBV after using HBIG frequently. In trial group, a few newborns were still HBeAg positive , but the HBV-DNA load had decreased obviously. It was likely the result of application of HBIG before labor. We presume that HBV-DNA load may decrease further if we keep on using HBIG and hepatitis B vaccine to those newborns with positive HBeAg. This deserves to study in the future. No side effect was found in both mothers and babies during the clinical trial.

Why were there still 7 cases of newborns with HBeAg positive in trial group, in our opinion., one of reasons was that cord serum used for HBeAg test was not better than vein serum, because cord serum is easy to be contaminated by the blood of mother with positive HBeAg. Another reason may be the high load of HBV-DNA in serum of these 7 mothers.

How to improve the effects of interruption of intrauterine transmission, other methods can be taken into consideration. Yue *et al*[10] used HBIG and together with hepatitis B vaccine to interrupt 30 pregnant women every 4 week from the 20th wk until delivery. The results showed that 29 newborn were HBsAg negative, 27 HBV-DNA negative and 10 were anti-HBs positive. In another chinical trial[11], 56 cases were given 200 IU HBIG intramuscularly every 4 wk from the 28th wk, while 43 cases received 100 mg lamivudine orally every day, 52 cases in the control group received no special treatment. The results demonstrated that the risk of HBV intrauterine infection effectively reduced in the 3rd trimester of HBsAg positive pregnant women, and there was no significant difference between HBIG and lamivudine groups. In present study, *Su et al*[12] has reported that 38 cases of pregnant women were given lamivudine before pregnancy, during pregnancy, until after labor. The result showed that none of 38 newborns was chronic carrier of HBsAg. In infant animals, lamivudine does not cause dysplasia however evidence of safety in human infants is limited. So some researchers prefer to use lamivudine from the 16th wk or 24th wk to those pregnant women with positive HBeAg for the safety of infants. We are waiting for the outcomes.

HBeAg is a marker which indicates active duplication of HBV, high concentration of HBV and strong possibility of infection. So we chose HBeAg positive pregnant women as subjects in this study. However it is unsuitable for clinical application to prevent from intrauterine transmission of HBV because the mutation of pre-C region of HBV can result in negative HBeAg , but they still had high load of HBV-DNA[13]. In clinical application, those pregnant women with negative HBeAg and positive HBV-DNA also need to be interrupted by HBIG. It has been reported that there is closed relationship between HBV-DNA load of serum of pregnant women and intrauterine infection[14,15]. Therefore it will be better to guide the application of prevention of mother-infant transmission of HBV by HBV-DNA load test quantitively.

It is clear that intrauterine infection is the main route of HBV transmission. According to present study, there is still a route of paternal-infants transmission of HBV[16]. Zhang *et al*[17]detected HBV markers of sperm of male patients with hepatitis B, HBV markers of peripheral blood of their partners and their newborns, the outcome was: HBV markers of mothers were negative, HBV markers of newborns were positive, situations were same as those of their fathers. It indicated that there was possibility of HBV transmission from farther to infant. Zhao *et al*[18] have detected HBV-DNA of sperma in male patients with hepatitis B, then a capture test of normal sperma was performed. The samples of infants from induced labor were tested for HBsAg and HBV-DNA, the result showed that HBV can cause vertical transmission via sperma. So in order to control intrauterine infection of HBV, we need to consider both mother and father. For noninfected pregnant women, while their partners are HBV carriers, application of pro-

phylaxis also should be considered in these fathers.

The limited number of HbsAG positive women and excluded cases may have produced a bias in our results warranting further study.

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RAPID COMMUNICATION

Study on biological characters of SGC7901 gastric cancer cell-dendritic cell fusion vaccines

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Key words: Biological character; SGC7901 cell; Gastric cancer cell; Dendritic cell; Fusion vaccine

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Abstract

AIM: To detect the biological characters of the SGC7901 gastric cancer cell-dendritic cell fusion vaccines.

METHODS: The suspending living SGC7901 gastric cancer cells and dendritic cells were induced to be fusioned by polyethylene glycol. Pure fusion cells were obtained by selective culture with the HAT/HT culture systems. The fusion cells were counted at different time points of culture and their growth curves were drawn to reflect their proliferative activities. The fusion cells were also cultured in culture medium to investigate whether they could grow into cell clones. MTT method was used to test the stimulating abilities of the fusion cells on T lymphocytes' proliferations. Moreover, the fusion cells were planted into nude mice to observe whether they could grow into new planted tumors in this kind of immunodeficiency animals.

RESULTS: The fusion cells had weaker proliferative activity and clone abilities than their parental cells. When they were cultured, the counts of cells did not increase remarkably, nor could they grow into cell clones in culture medium. The fusion cells could not grow into new planted tumors after planted into nude mice. The stimulating abilities of the fusion cells on T lymphocytes' proliferations were remarkably increased than their parental dendritic cells.

CONCLUSION: The SGC7901 gastric cancer cell-dendritic cell fusion vaccines have much weaker proliferative abilities than their parental cells, but they keep strong abilities to irritate the T lymphocytes and have no abilities to grow into new planted tumors in immunodeficiency animals. These are the biological basis for their anti-tumor biotherapies.

INTRODUCTION

With the development of modern immune research, biotherapy has brought new hopes for gastric cancer patients to conquer this kind of malignant tumors[1]. The anti-tumor vaccines based on dendritic cells (DC) play important roles in anti-tumor biotherapies[2]. Researchers obtain the cancer cells' antigens by various methods to irritate the dendritic cells, and increase the antigen presenting and immune stimulating abilities of dendritic cells[3,4]. Researchers usually destroy the cancer cells by frozen or ultrasound[5] methods. The components of the destroyed cancer cells were used as cancer antigens to irritate the dendritic cells. Many other researchers transmitted the cancer cell related mRNA into dendritic cells [6] or mix-cultured the cancer cells and dendritic cells[7]. They also attempted to improve the antigen presenting and immune stimulating abilities of dendritic cells by these methods. Although all these methods could partially enhance dendritic cells' capabilities and anti-tumor immune function of cancer patients, they still have some limitations. In recent years, methods to induce the dendritic cells and cancer cells to be fusioned together have been developed in order to investigate whether the fusion cells have stronger immune stimulating abilities than normal dendritic cells. Gastric cancer cells have no strong and specific tumor antigens, which makes it difficult for gastric cancer biotherapies. If gastric cancer cells and dendritic cells could be fusioned together, the cancer cell antigens might be presented more efficiently and more obvious anti-tumor effects might be achieved. In this research, the gastric cancer cells and dendritic cells were induced to be fusioned together by cell fusion technology. We detected the fusion cells' biological characters so to provide experimental data for gastric cancer biotherapies.

MATERIALS AND METHODS

Reagents

The reagents used included rhGM-CSF (Promega Co. Ltd, USA), rhIL-4 (R&D System Co. Ltd), TNF-a(R&D System Co. Ltd), Polyethylene glycol (Sigma, USA), Hypoxanthine (Sigma, USA), Aminopterin (Sigma, USA), Thymidine (Sigma, USA).

Preparation for suspending SGC7901 gastric cancer cells and dendritic cells

The frozen stored SGC7901 gastric cancer cells were cultured in the DMEM culture medium, and the suspending living cancer cells were prepared for the fusion process. The peripheral blood mononuclear cells were separated from venous blood of gastric cancer patients by use of lymphocytes separating solution. rhGM-CSF (100 ng/mL), rhIL-4 (50 ng/mL) and TNF-α(20 ng/mL) were added into the cultured peripheral blood mononuclear cells to induce them to transform into dendritic cells.

Preparation for SGC7901 gastric cancer cell-dendritic cell fusion vaccines

Suspending living SGC7901 gastric cancer cells and dendritic cells were mix-cultured with the ratio of 10:1. The two kinds of parental cells were induced to be fusioned by polyethylene glycol and selectively cultured by HAT/HT culture solutions. After the culture process, we obtained pure fusion cells as anti-tumor vaccines. Simultaneously, the suspending living SGC7901 gastric cancer cells and dendritic cells were mix-cultured with the same ratios, but were not induced to the fusion process. This group was designed as control group.

Growth curves of fusion cells

The obtained pure fusion cells (cell density 1.2×10^6/mL) were inoculated into the culture media on the culture slabs. During the culture process, we counted the cell numbers daily. After fourteen days, we drew the growth curves of cells. The SGC7901 cancer cells' and dendritic cells' growth curves were drawn at the same time respectively, and these curves were served as control.

Cloning abilities of fusion cells

The suspending living fusion cells (cell density 1×10^4/mL) were inoculated into the agar culture medium on twenty-four holes culture slabs. The cell numbers in each hole were 1000, 2000 and 10 000 respectively. After two weeks' cultivation, the cell clones which included more than 50 cells were counted under the microscopes. At the same time, the clone abilities of SGC7901 gastric cancer cells and dendritic cells were tested by the same methods.

Abilities of fusion cells to stimulate T lymphocyte proliferation

T lymphocytes were separated from peripheral blood of gastric cancer patients by using nylon hair. The cell densities of T lymphocytes, fusion cells and mix-cultured dendritic cells were adjusted to 5×10^6/mL. The fusion cells and mix-cultured dendritic cells were cultured on the culture slabs with 96 holes. T lymphocytes were added. The ratios of T lymphocytes to fusion cells and mix-cultured dendritic cells were 100:1, 50:1, 10:1 and 1:1 respectively. For each ratio, six holes were repeated in order to decrease the systemic errors. After sixty hours' cultivation, ^{3}H-TdR was added into each hole on the slabs (1Ci per hole). Twelve hours later, the CPM values were tested by Beckman liquid scintillation counting. The culture groups which included T lymphocytes only or DMEM culture medium were designed as control groups.

Abilities of fusion cells to grow into new planted tumors in nude mice

The suspending living pure fusion cells (cell density 1×10^9/mL, cell number 5×10^8) were planted subcutaneously into the back of the BALB/c nude mice. The injections were performed every three days for about two weeks. We observed whether the planted fusion cells could grow into new planted tumors in the nude mice, and the percentages of planted tumor cells growing into new planted tumors were tested. At the same time, the SGC7901 gastric cancer cells were planted into other groups of nude mice. These groups were designed as control groups.

Statistical analysis

Data were imported into Systat software (SPSS) for statistical computations and graphing. One-way analysis of variance (ANOVA), independent-samples T test and Chi-Square tests were used to evaluate the differences between groups. $P < 0.05$ was considered as significant.

RESULTS

Growth curves of fusion cells

We obtained the fusion cells after the selective culture process. The fusion cells' phenotypes and morphological characters were examined by flow cytometer and histological methods. The parental SGC7901 gastric cancer cells proliferated fast during their cultivation. After the subculture, the cancer cells stayed in proliferative delitescence for a short period, and then they entered into the logarithmic growth period with cell number increasing quickly. The cancer cells' proliferation slowed down and their number decreased gradually (Figure 1). Dendritic cells had much weaker proliferative abilities than SGC7901 cancer cells. During their cultivation, the cells' number did not increase remarkably. Like the parental dendritic cells, fusion cells also had weak proliferative abilities with a flat growth curve. This indicated that fusion cells' proliferative characters were much similar to parental dendritic cells (Figure 2).

Cloning abilities of fusion cells

Clone abilities effectively reflect the proliferation of single cells. If a kind of cell could grow into numerous cell clones in the agar culture medium, it indicates these cells have strong abilities to proliferate. In our study, we found that SGC7901 gastric cancer cells had strong proliferative abilities. They grew into numerous cell clones fast. Like the parental dendritic cells, fusion cells also had much weaker proliferations. During their cultivation, they could not

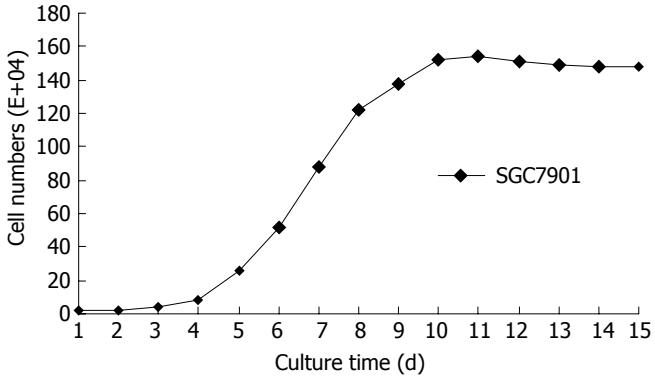


Figure 1 The growth curve of SGC7901 gastric cancer cells.

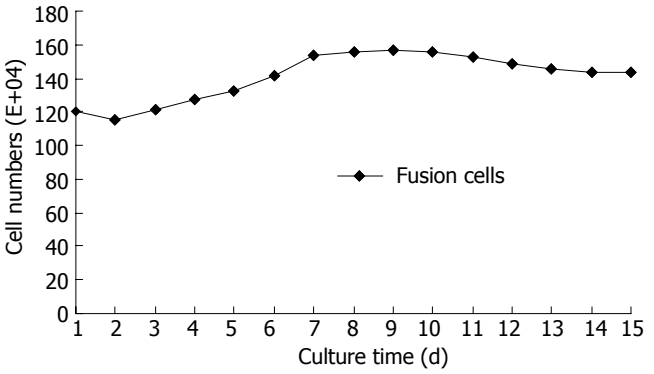


Figure 2 The growth curve of SGC7901-DC fusion cells.

grow into visible cell clones.

Abilities of fusion cells to irritate T lymphocytes' proliferation

The abilities of fusion cells to irritate T lymphocytes were much stronger than the mix-cultured DC group ($P < 0.01$) (Table 1).

Abilities of fusion cells to grow into new planted tumors in nude mice

After injected subcutaneously into the back of the BALB/c nude mice, the SGC7901 gastric cancer cells grew into new planted tumors quickly, whereas the fusion cells could not grow into new tumors in nude mice, even with a higher cell density and injection frequency, suggesting that fusion cells had no abilities to proliferate and grow into new planted tumors in immunodeficiency animals.

Table 1 Abilities of different cells to irritate T lymphocytes' proliferations (mean ± SD, CPM)

Groups	1:1	1:10	1:50	1:100
Fusion vaccine group	1 5083 ± 231[1]	9608 ± 83[2]	4214 ± 135[3]	3020 ± 28[4]
Mix-cultured DC group	5977 ± 272	3019 ± 38	2086 ± 58	1500 ± 41

[1]$P = 0.005$, [2]$P = 0.003$, [3]$P = 0.002$, [4]$P = 0.003$ *vs* Mix-cultured DC group.

DISCUSSION

Characters of fusion cells' growth curves, cloning abilities in vitro and their biological significance

Cell number and growth curves could directly reflect cell proliferative characters[8]. Clone ability is an important criterion to judge cells' growing capabilities[9]. The stronger the growth powers, the more cell clones could be formed. Cancer cells had strong abilities to proliferate. In our research we found that when the SGC7901 gastric cancer cells were subcultured, they quickly grew into the logarithmic proliferation period and the cell number increased remarkably[10]. After a short period the cell number was doubled. They grew into clearly cell clones only after seven days' cultivation. On the contrary, dendritic cells had much weaker proliferative abilities with a flat growth curve. There were no remarkable increases of the cell number during their cultivation, nor did cell clones formed. We found that fusion cells' growth characters were similar to their parental dendritic cells. They also had much weaker proliferative abilities and could not grow into cell clones during their cultivation. The cell number maintained a stable level and the growth curves were just a flat line. The SGC7901-DC fusion cells kept more biological characters of their parental dendritic cells. Different from their parental cancer cells, the fusion cells had no abilities to proliferate fast and grow into cell clones. These indicate that fusion cells were safe to be used as anti-tumor vaccines, which are biological basis for fusion cells research in cancer treatment.

Characters of fusion cells to irritate T lymphocyte's proliferation and their biological significance

After tumor antigens were presented by the dendritic cells, T lymphocytes were irritated and showed anti-tumor biological effects[11]. T lymphocytes *in vivo* can be divided into several sub-groups which have different phenotypes and biological effects. The most important sub-groups are CD4+ T lymphocytes (Th) and CD8+ T lymphocytes (Tc/CTL)[12]. The in-activated pre-Tc cells were irritated and became active Tc cells *in vivo* by the tumor antigens and CD4+ Th cells. These activated Tc cells exerted anti-tumor biological effects by the double signal approaches[13]. The cell number and activities of T lymphocytes directly affected anti-tumor immune responses of cancer patients[9]. Our purposes of modifying dendritic cells as anti-tumor vaccines were to increase their abilities of stimulating T lymphocytes' proliferation and their activities[14,,15]. Some researchers reported that when the tumor antigen related genes were transmitted into dendritic cells T lymphocytes *in vivo* could be strongly irritated to become CD4+ Th cells and CD8+ Th cells [16,17]. In our research, we found that the SGC7901-DC fusion cells could strongly irritate T lymphocytes' proliferation, while the mix-cultured dendritic cells could not. This indicated that fusion cells had much stronger abilities to activate anti-tumor immune responses. So, fusion cells are possible to be used as anti-tumor vaccines.

Abilities of fusion cells to grow into new planted tumors in immunodeficiency animals and their biological significance

New planted tumors in immunodeficiency animals are

perfect models for oncology researches[18]. We injected the SGC7901 cancer cells and SGC7901-DC fusion cells into different groups of nude mice and found that SGC7901 cancer cells could grow into new planted tumors quickly but the fusion cells could not, even when much higher cell densities and more injection frequencies were applied. This implied that SGC7901-DC fusion cells had no abilities to grow into new planted tumor in nude mice. This is the most important difference between the parental SGC7901 cells and the SGC7901-DC fusion cells. This character together with the growth curves and clone abilities indicated that fusion cells were safe when used as anti-tumor vaccines *in vivo* and *in vitro*.

The strong abilities of SGC7901-DC fusion cells to irritate T lymphocytes' proliferation and the characters that the fusion cells could not grow into new planted tumors *in vivo* are the precondition of anti-tumor biological therapy.

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CASE REPORT

Granular cell tumor of stomach: A case report and review of literature

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Abstract

Granular cell tumor (GCT) was described for the first time by Abrikosoff in 1926. It is a relatively rare neoplasm that may occur at many sites, but most commonly in the skin or soft tissues. The occurrence of GCT in the gastrointestinal tract is rare, accounting approximately for 8% of all tumors, among which the most common site is the esophagus, whereas gastric localization is very rare. Gastric GCTs can be solitary or, more frequently, associated with other gastrointestinal localization. Although GCTs are usually clinically and histologically benign, some malignant cases have been reported. Histologically, these tumors consist of polygonal and fusiform cells disposed in compact "nests" and immunohistochemical staining for S-100 protein supports the proposed derivation from Schwann cells. A correct preoperative diagnosis of this tumor can only be made in 50% of all patients and it is always based on endoscopic biopsy. Laparoscopic or conventional wedge resection represents the treatment of choice. In this study, the authors reported a case of a 49-year-old woman with a solitary granular cell tumor of the stomach with infiltrative pattern, successfully treated with surgical resection. A review of literature is also presented with emphasis on diagnostic criteria concerning the malignant form.

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Key words: Granular cell tumor; Stomach; Benign; Surgical resection

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INTRODUCTION

Granular cell tumors (GCT) were described for the first time by Abrikosoff in 1926. They are uncommon, usually benign, soft tissue tumors rarely seen in clinical practice. They may occur at many sites, although it affects most frequently skin or subcutaneous tissues of the chest and upper extremities, tongue, breast and female genital region. The onset of this type of tumor in the gastrointestinal (GI) tract is rare. Approximately 8% of all GCTs occur in the digestive tract[1] and among these the most common site is the esophagus, followed by the large intestine[2]. Gastric localization is seldom seen. Yasuda et al[3], reported a case of gastric GCT in 1995 and underlined that this lesion was previously found in the stomach only in 24 patients. Since 1996, five more cases have been described[4-9].

In this study, we report a case of a woman with a solitary GCT of the stomach, incidentally found upon GI endoscopy, which had an infiltrative growth pattern and was successfully treated by surgical resection.

CASE REPORT

A 49-year-old woman was admitted to our Unit for further evaluation of a gastric mass which had been incidentally in an abdominal ultrasound examination for her nonspecific dyspeptic symptoms (Figure 1). She had no remarkable past medical history, and no significant alcohol consumption or history of smoking or drugs. Upon hospitalization, physical examination, biochemical data and tumor markers (CEA, CA-19-9) were all within normal limits. Upper gastrointestinal endoscopy was performed and revealed a hemispherical mass of about 3 cm in diameter, located on the lesser curvature of the gastric antrum and overlaid by normal mucosa. Search for *H pylori* by biopsy urease (Pantorc® *Hp* Test, Byk Gulden Italia S.p.A.) was negative. The esophagus, duodenum and the remaining parts of stomach were normal. Multiple deep (jumbo) biopsies from the gastric mass and from the surrounding gastric mucosa were performed. Histopathological examination of mass biopsies showed a pattern compatible with GCT, whereas other mucosal biopsies were normal. A subsequent CT scan of the abdomen showed a solid mass, with a slight contrast enhancement, of about 3 cm in diameter with well-defined margins located near the gastric angulus, arising from a layer of the gastric wall. Neither further invasion beyond the gastric wall, nor visible metastatic lesions in the liver or enlarged lymph nodes were observed. During laparotomy a tumor mass was found on the anterior wall of the lesser



Figure 1 Echo tomography of gastric region. A solid hypoechogenic mass with a diameter of 2.7 cm × 2.2 cm protruding from beyond the anterior wall of the stomach.



Figure 2 Macroscopic appearance of resected specimen: section of surgical specimen: a soft yellow mass of about 2 cm in diameter.

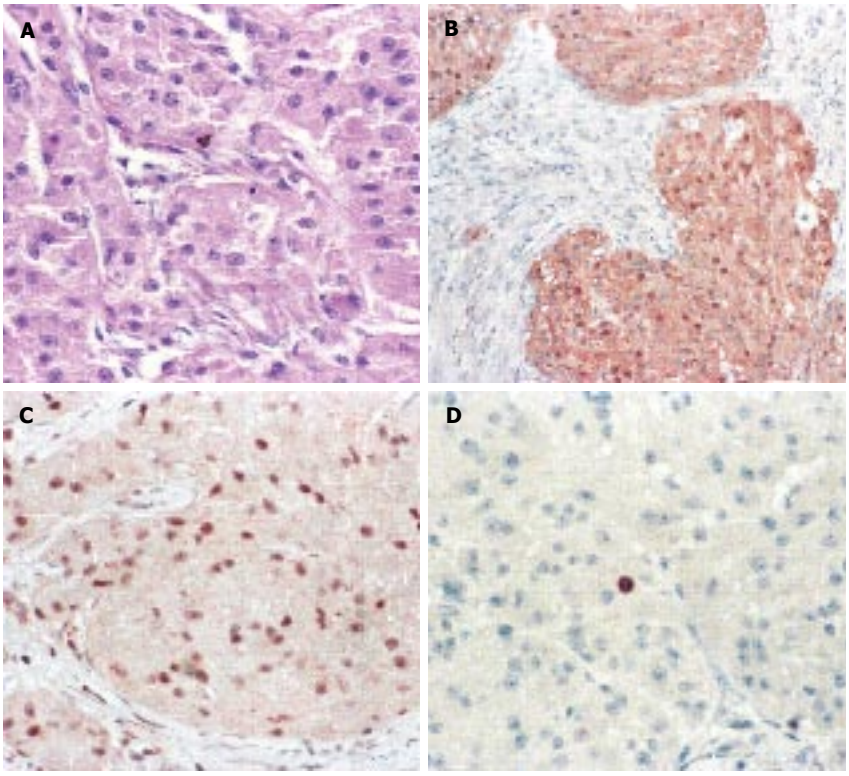


Figure 3 Histological findings of the resected specimen. **A:** hematoxylin–eosin staining. Sheet of large and polygonal cells with round to oval, eccentrically located nuclei and eosinophilic granular cytoplasm with lower mitotic activity; **B**: immunohistochemical staining for S-100 protein. Diffuse and strong expression of S-100 protein; **C**: immunohistochemical staining for p53. Some of the tumor cells are positive for p53; **D**: immunohistochemical staining for Ki-67. Some of the tumor cells are positive for Ki-67.

curvature of the stomach. There were no liver metastases or regional lymph node swelling. A wedge resection, with a gastric section performed at a distance of about 1.5 cm from the macroscopic margin of the tumor, was carried out, obtaining a total removal of the lesion.

In the resected specimen, the tumor measured 2 cm in diameter and appeared as a yellow mass (Figure 2). Microscopically, the tumor was located mainly in the muscle layer of the stomach. It formed solid nests of cells ranging from round to polygonal shape associated with few spindle cells. The nuclei were of different sizes with nucleoli at times evident and finely dispersed chromatin. The cytoplasm was abundant and finely granular. Mitotic activity was very low. The tumor focally infiltrated near muscular layer but did not affect mucosal surface (Figure 3A). Immunohistochemistry showed, S-100 protein present nearly in all cells within the tumor mass (Figure 3B), p53 in 40% of cells (Figure 3C) and Ki-67 in less than 9% of cells (Figure 3D). These histological findings met the criteria for a diagnosis of benign GCT[10].

The postoperative course was uneventful and the patient was discharged on postoperative fifth day. She remained asymptomatic and no recurrent disease was observed after a two year follow-up.

DISCUSSION

From an extensive review of literature about gastric GCT, no differences of tumor incidence was found as regard to gender, whereas fourth through sixth decades of life seem to be more affected. In term of ethnical issue of affected patients, the most reported cases were described in Japanese population. Moreover, in 50% of cases, gastric GCT proved to be associated with esophageal synchronous localization and was rarely associated with other benign or malignant gastric diseases. In only two reported patients,

Table 1 Characteristics of reported gastric granular cell tumors since 1998

Authors (yr)	Country	Age (yr)	Gender	Tumor size (cm)	Gastric site	Simultaneous occurrences of GCT	Simultaneous gastric lesions	Treatment
Sebastian (1998)	Spain	53	M	7	Upper	Esophagous	Ulcers	Surveillance
David (1999)	USA	45	F	2	Upper	Esophagous	No other lesions	Surgical
Eguchi (2002)	Japan	64	M	1.5	Body	No other occurrences	Cancer and lymphoma	Surgical
Maekawa (2003)	Japan	53	M	NR	Upper multiple	Esophagous	No other lesions	Surgical
Sailors (2005)	USA	65	F	1.5	n.r.	Tranverse colon	Cancer	Surgical

NR: non reported; GCT: granular cell tumor.

similarly to our case, gastric GCT did not show multiple localizations or was associated with other gastric lesions[3,11] (Table 1).

These gastric tumors are mostly confined macroscopically to submucosa. Their size may range from few millimeters to some centimeters, never surrounded by capsule. Histologically, gastric GCTs are composed by polygonal and fusiform cells arranged as compact "nests". Immunohistochemical staining for S-100 protein supports the proposed origin of the tumor from Schwann cells[12].

Although GCTs are usually benign clinically and histologically, some malignant cases have been reported. There are less than 30 reported cases of malignant GCTs in world literature[13] and the only documented case of a malignant GCT of the stomach was reported in 1996 by Matsumoto *et al*[9] (Table 1).

Features associated with malignancy include local recurrence, rapid growth to a size greater than 4 cm, cell necrosis, spindling of tumor cells, cytologic atypia and high mitotic activity, vescicular nuclei with large nucleoli, and a high nuclear-to-cytoplasm ratio[5,10]. It has also been reported that, on immunohistochemical staining, positivity rate of more than 50% for p53 and more than 10% for the Ki-67 index respectively, was significantly correlated with malignancy[7,10]. Extent of infiltration[5] or highly developed tumor microvessels[14] or focal pleomorphism[7] have never been considered as a criterion for diagnosis of malignancy. Altogether, these features are suggestive for malignant potential of the tumor. The tumor mass in our patient infiltrated only focally and marginally the muscular layer and did not display high expression of p53 and Ki-67. Furthermore, after the follow-up for two years, no evidence of recurrences was seen.

It is questionable whether these tumors always produce GI symptoms[15], even when they are of small size. It is quite likely that, if small, a change (pre-symptomatic) was found during upper GI endoscopy, X-ray or ultrasound examination performed for nonspecific dyspepsia. When large, gastric GCTs may instead present as gastric outlet obstruction[11], or massive upper GI hemorrhage[16].

A correct preoperative diagnosis of this tumor can be made by endoscopic biopsy. It is generally accepted that the best way to obtain an appropriate sampling of such tumors beneath the mucosa is either by boring or by performing a jumbo biopsy. A definitive diagnosis can only be established by endoscopic biopsy in 50% of the patients[17]. Endoscopic ultrasonography has recently been used more frequently for determining the depth of tumor invasion in the gastrointestinal wall, and may also be useful to evaluate GI tract submucosal tumors[2]. Endoscopic excision of GCT should be strongly considered[3]. If the tumor is attached to muscularis propria, an injection of saline can be useful to increase the distance, so that endoscopic removal can be performed more safely[3]. Therefore, since the gastric GCT is without capsule and focal areas of infiltration of the gastric wall can be present, laparoscopic or conventional wedge resection, including at least one centimeter of normal tissue, is the best therapeutic option. Laparoscopic approach could be difficult due to tumor localization beneath the posterior wall or lesser curvature, or mass size (very large or very small). Almost all gastric GCTs, observed from 1990 to date, were treated with conventional surgical approach.

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CASE REPORT

Primary signet-ring cell carcinoma of the colon at early stage: A case report and a review of the literature

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Abstract

A 67-year-old man, who had undergone surgery to resect multiple gastric cancers 4 years ago, visited our hospital for surveillance colonoscopy. Colonoscopy revealed a discolored, 7-mm in diameter, flat-elevated lesion with central depression in the transverse colon near the splenic flexure. Although the findings of endoscopy and barium enema were suggestive of submucosal invasion, the patient chose to undergo endoscopic mucosal resection. Pathological examination of the resected specimen revealed signet-ring cell carcinoma and a positive surgical margin. A second operation was performed, and no residual tumor or metastasis to lymph nodes was found in the resected specimens. Primary colorectal cancers composed of signet-ring cell carcinoma detected and treated at an early stage are extremely rare. We present a case and review the literature.

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Key words: Signet-ring cell carcinoma; Early colorectal cancer; Endoscopic mucosal resection

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INTRODUCTION

Signet-ring cell carcinoma is uncommon in the colon and rectum, with a reported incidence ranging from 0.1% to 0.9%[1,2]. In general, signet-ring cell carcinoma shows the characteristic appearance of "linitis plastica" and behaves more aggressively than carcinoma of other histological types[1,2]. As symptoms usually appeared late, signet-ring cell carcinomas are commonly detected at advanced stages. Therefore, cases detected and treated at early stage are rare. In this case report, we present our experience with a signet-ring cell carcinoma in the transverse colon detected at early stage and review the literature.

CASE REPORT

A 67-year-old man visited our hospital 4 years ago with a history of 4 synchronous primary cancers of the stomach. He underwent total gastrectomy, and the surgical specimen contained 2 early cancers which invaded the mucosa and submucosa, and 2 advanced cancers which penetrated serosa without invasion of adjacent structures. No components of signet-ring cell carcinoma were found on histology. He had a brother and a sister, and the family history included that his father had died of a gastric cancer at the age of 68, and his sister had an advanced sigmoid colon cancer and a uterine cancer at the age of 62 and 65, respectively. The patient's past history included an operation for synchronous gastric cancers, however there was no history of inflammatory bowel disease. He also had a focal cancer in an adenoma with moderate atypia, which had been completely resected endoscopically one year before. Therefore, he underwent surveillance colonoscopy at our department of endoscopy. Findings on laboratory tests were unremarkable. Total colonoscopy, with a magnifying colonoscope (CF-200Z, Olympus Optical Co., Japan) was easily introduced into the cecum without complications. Colonoscopy revealed a discolored flat-elevated lesion with central depression, 7 mm in diameter, in the transverse colon near the splenic flexure (Figure 1A). The tumor bled easily from the depressed area without contact from the endoscope and had convergent mucosal folds suggesting submucosal cancer involvement. After spraying of 0.2% indigo-carmine dye, the depression was seen more clearly (Figure 1B). After chromoendoscopy with 0.2% indigo-carmine dye and 0.05% crystal violet staining, we attempted to observe the surface structure of this

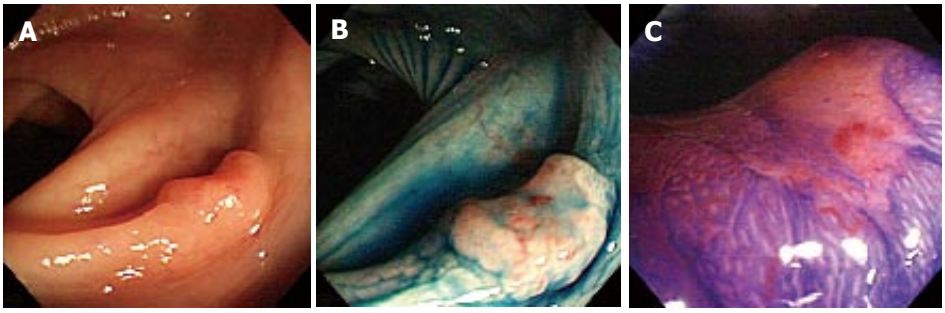


Figure 1 A: Colonoscopy revealed a discolored, flat-elevated lesion with central depression, 7 mm in diameter, in the transverse colon near the splenic flexure; **B:** Chromoendoscopy with 0.2% indigo-carmine dye showed the depressed area more clearly; **C:** Magnifying colonoscopy after 0.05% crystal violet staining was attempted but failed to detect the pit pattern for depth estimation, as the depressed area was covered by dense mucus, which could not be removed by repeated water washing.

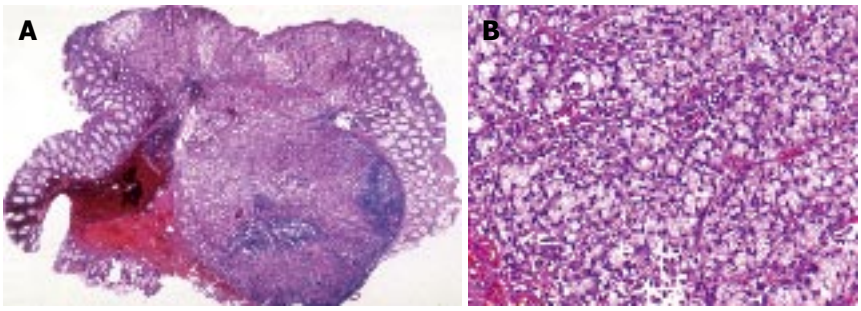


Figure 2 A, B: Histologically, the resected specimen showed a signet-ring cell carcinoma that had deeply invaded into the submucosal layer, and the vertical cut end of the resected tumor was positive for carcinoma cells.

lesion, the so-called "pit patterns", at a higher magnification to estimate the depth of invasion[3,4]. However, the pit patterns could not be detected, as dense mucus coated the surface and could not be removed by repeated washing (Figure 1C)[5]. The findings of barium enema and conventional colonoscopy suggested submucosal cancer invasion, and thus, surgical resection, including node dissection, was recommended for curative treatment. However, the patient refused surgical resection but consented to endoscopic mucosal resection (EMR). The lesion was successfully removed en bloc with EMR without complications. The resected specimen was 7 mm in diameter. The resected specimen was fixed with 20% formalin, and then the mucus on the surface was removed for better staining and observation. The fixed lesion was stained with 0.05% crystal violet and observed stereomicroscopically under water immersion. Stereomicroscopy revealed a type V (non structural) pit pattern in the depressed area. These findings suggested that this lesion had deeply invaded the submucosal layer[3,6]. Histologically, the tumor was composed of signet-ring cell carcinoma, without accompanying adenoma or other types of cancer cells, that had infiltrated deeply into the submucosal layer with invasion of lymphatic vessels (Figure 2A-B). Immunostaining was used to examine the expression of MLH1 and MSH2 of the resected specimen; the tumor was positive for MSH2 but negative for MLH1. A cut end of the resected specimen was positive for carcinoma cells. Systemic diagnostic imaging including abdominal computed tomography showed no evidence of distant metastasis or ascites. Thereafter, the patient consented to undergo an additional laparotomy. The resected specimen revealed no residual carcinoma at the EMR site and showed no metastasis to lymph nodes. The postoperative course was uneventful, and the patient is still alive and in apparent good health 5 years after the operation.

DISCUSSION

More than 96% of signet-ring cell carcinomas arise in the stomach, and the rest occurred in other organs, including the colon, rectum, gallbladder, pancreas, urinary bladder, and breast[7]. The case described here was believed to be a primary lesion of the transverse colon because a thorough workup did not reveal any other sites of involvement. Although the patient's history included surgical resection of an advanced gastric carcinoma, a secondary carcinomatous deposit should also be excluded. Metastasis from gastric carcinoma is probably the most common secondary carcinoma in the colon and rectum[8]. It may present itself as single or multiple strictures mimicking primary colorectal carcinoma or inflammatory bowel diseases[8,9]. Furthermore, multiple flat and depressed lesions or polyps have also been reported as rare presentations of colonic metastasis from gastric signet-ring cell carcinoma[10-13]. Our case presented a solitary depressed lesion without metastasis or lymphatic, hematogenous or peritoneal involvement, and the patient is still alive 5 years after surgery; therefore, on the basis of these findings, this case could be judged as a primary lesion. Crystal violet staining is commonly used to evaluate pit patterns, especially for lesions in which there is the suspicion of submucosal invasive carcinoma. Unfortunately, the pit patterns could not be detected in this case, as dense mucus coated the surface and failed to be removed by repeated washing endoscopically. This might reveal that signet-ring cell carcinoma produces abundant mucus.

Primary signet-ring cell carcinoma of the colon and rectum, as first described by Laufman and Saphir in 1951, is rare[14]. The reported incidence is about 1%[1,2]. The histological appearance of the tumor is characterized by cells with abundant intracytoplasmic mucin and peripherally placed nuclei. Signet-ring cell carcinomas of the colon and

rectum are usually diagnosed at an advanced stage, because symptoms usually develop late. Thereafter, cancers limited to the mucosal and submucosal layers are rarely detected; to the best of our knowledge, only 26 cases, including ours, have been reported[15-20]. The clinicopathological details of the reported cases are summarized in Table 1. The patients included 21 males and 5 females with a mean age of 57.1 years (range of 6-79 years). The mean size of the tumors was 16.0 mm (range, 2 to 45 mm). Macroscopically, 16 (61.5%) cases were flat or depressed. The smallest tumor was a tiny, discolored, flat lesion 2 mm in diameter. Despite the small size, it surprisingly metastasized to lymph nodes[18]. Tsujinaka et al have described the first case of early signet-ring cell carcinoma of the colon without an adenomatous component. It was a depressed lesion that was believed to have arisen via a de novo pathway[15]. This tumor only showed signet-ring cancer cells without accompanying adenoma or other types of cancer cells. It is unknown whether signet-ring cell carcinoma usually arises from a pre-existing adenomatous polyp or as a so-called de novo carcinoma[21]. However, according to the reviewed articles, there are 3 reported cases with an adenomatous component; therefore, an adenoma-signet-ring cell carcinoma sequence also exists[16,17].

The sites of reported cases are mostly in the right-sided colon (50%, 13 cases), but 7 are in the left-sided colon and 6 are in the rectum (Table 1). In our case, the transverse colon was the most commonly involved site. Eight of the reported cases also had multiple synchronous colorectal cancers. The right-sided predilection, histological characteristics, and the tendency to multiplicity are now well documented in hereditary non-polyposis colorectal cancer. However, in the present case, the family history and past history did not fulfill the diagnostic criteria[22,23]. Considering the clinicopathological characteristics of this case, immunostaining was used to examine expression of MLH1 and MSH2 of the resected specimen; the tumor was positive for MSH2 but negative for MLH1. Signet-ring cell carcinomas have been analyzed for microsatellite instability, which is present in approximately 30% of tumors[24]. Furthermore, although not available in our case, mutations of K-ras and p53 gene have been reported in signet-ring cell carcinomas of the colon and rectum; however, the frequency of K-ras gene mutation in signet-ring cell carcinomas is significantly lower than that of well and moderately differentiated carcinomas[24]. These results suggest that the genetic background of signet-ring cell carcinomas might differ from that of well or moderately differentiated carcinomas of the colon and rectum. DNA-replication errors are suggested to be at least partly involved in the carcinogenesis of signet ring cell colorectal carcinoma[24]. Although, our patient did not have inflammatory bowel disease, an association of inflammatory bowel disease and signet-ring cell carcinoma of up to 14% has been reported[25]. Psathakis et al. reported two (14.3%) of 14 patients with signet-ring cell carcinoma, who had a long history of ulcerative colitis[26]. Anthony et al. reported two cases of Crohn's disease that developed signet-ring cell carcinoma[27].

In conclusion, we have reported a rare case of primary signet-ring cell carcinoma of the colon detected and treated at early stage, and reviewed the literature.

Table 1 Clinicopathological details in patients with primary early colorectal signet ring cell carcinomas

Mean age (range) (yr)	57.1 (6-79)
Sex (male/female)	21/5
Mean size (range) (mm)	16.0 (2-45)
Site of lesions	
Right-sided colon (cecum/ascending/transverse)	13 (3/1/9)
Left-sided colon (descending/sigmoid)	7 (3/4)
Rectum	6
Depth of invasion	
Intramucosal	6
Submucosal	20
Macroscopic features	
Polypoid	10
Flat	3
Depressed	13
Lymph node metastasis	
Positive	2
Negative/unknown	17/7

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CASE REPORT

Postoperative bile leakage managed successfully by intrahepatic biliary ablation with ethanol

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Abstract

We report a case of postoperative refractory bile leakage managed successfully by intrahepatic biliary ablation with ethanol. A 75-year-old man diagnosed with hepatocellular carcinoma underwent extended posterior segmentectomy including the caudate lobe and a part of the anterior segment. The hepatic tumor attached to the anterior branch of the bile duct was detached carefully and resected. Fluid drained from the liver surface postoperatively contained high concentrations of total bilirubin, at a constant volume of 150 mL per day. On d 32 after surgery, a fistulogram of the drainage tube demonstrated an enhancement of the anterior bile duct. Endoscopic retrograde cholangiography demonstrated complete obstruction of the proximal anterior bile duct and no enhancement of the peripheral anterior bile duct. On d 46 after surgery, a retrograde transhepatic biliary drainage (RTBD) tube was inserted into the anterior bile duct under open surgery. However, a contrast study of RTBD taken 7 mo post-surgery revealed that the fistula remained patent despite prolonged conservative management, so we decided to perform ethanol ablation of the isolated bile duct. Four mL pure ethanol was injected into the isolated anterior bile duct for ten minutes, the procedure being repeated five times a week. Following 23 attempts, the volume of bile juice reached less than 10 mL per day. The RTBD was clamped and removed two days later. After RTBD removal, the patient had no complaints or symptoms. Follow-up magnetic resonance imaging demonstrated atrophy of the ethanol-injected anterior segment without liver abscess formation.

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Key words: Postoperative complication; Bile leakage; Ethanol ablation

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INTRODUCTION

Bile leakage is one of the major postoperative complications that may occur after hepatectomy. The reported incidence of postoperative bile leakage ranged from 4.8% to 7.6%[1-3]. Biliary complications including bile leakage after hepatic surgery are a major cause of morbidity and extended hospital stays. Lo *et al*[4] reported a high mortality rate in patients who required reoperation for biliary complication after liver resection. Biliary drainage is usually important and necessary for the treatment of bile leakage, but sometimes fails to improve bile leakage despite prolonged conservative drainage[5].

We report a case of postoperative refractory biliary leakage managed successfully by intrahepatic biliary ablation with ethanol.

CASE REPORT

A 75-year-old man was referred to our hospital with a diagnosis of hepatic tumor. Abdominal computed tomography confirmed a 63 mm × 59 mm hepatic mass locating mainly in segment I with invasion into segment VI, VII, VIII, and V. This tumor obstructed the posterior branch of the bile duct, and oppressed the anterior branch of the bile duct, middle hepatic vein and infra vena cava (Figure 1A, B). Abdominal angiography revealed a hypervascular tumor in the right lobe at the early enhanced phase (Figure 1C). Serum alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) was 227 ng/mL (normal, < 20) and 62 mAU/mL (normal, < 40), respectively. He had no additional underlying liver disease. We diagnosed him as hepatocellular carcinoma and decided upon surgery for radical treatment. An extended posterior segmentectomy including the caudate lobe and a part of the anterior segment was performed. Consistent

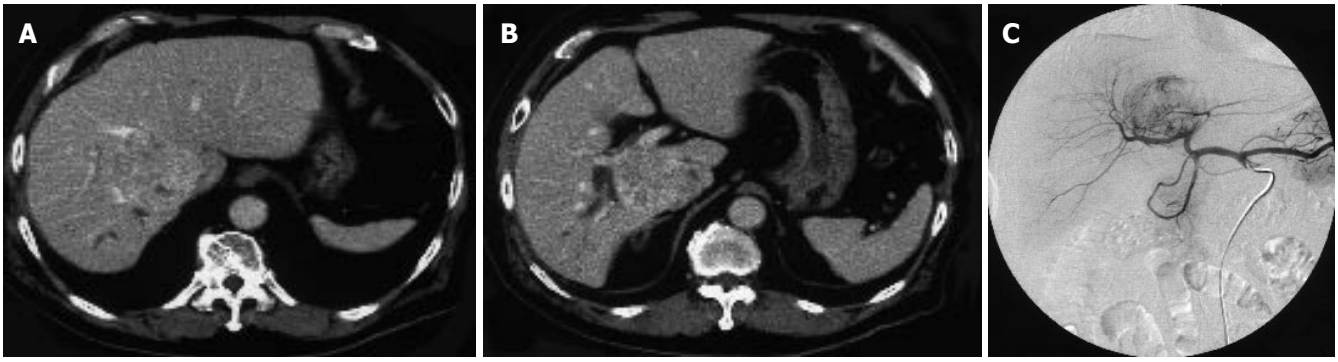


Figure 1 **A**, **B**: Abdominal computed tomography confirmed a 63 mm × 59 mm hepatic mass locating mainly in segment I with invasion into segment VI, VII, VIII, and V. This tumor obstructed the posterior branch of the bile duct, and oppressed the anterior branch of the bile duct; **C**: Angiography revealed a hypervascular tumor in the right lobe at the early enhanced phase.

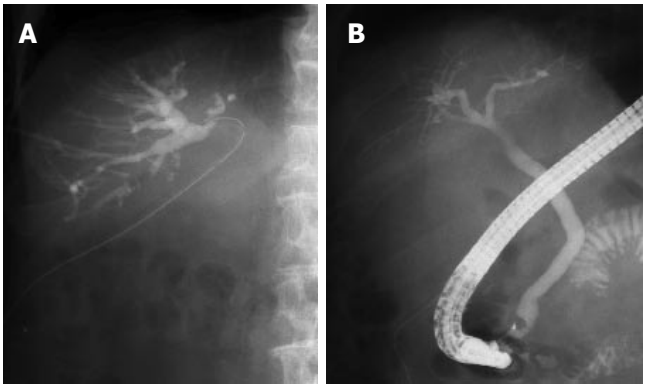


Figure 2 **A**: A fistulogram from the drainage tube demonstrated a connection between the anterior bile duct and fistula; **B**: Endoscopic retrograde cholangiography showed obstruction of the anterior bile duct, and no connection between the common bile duct and the anterior bile duct.

with the findings of the preoperative computed tomography, the tumor was attached to the anterior branch of the bile duct, middle hepatic vein, and inferior vena cava, and was detached carefully from them and resected. During surgery, no particular problems were seen, including bile leakage. Postoperatively, the fluid from a drain locating in the resected liver surface contained total bilirubin (61.2 mg/dL), the volume of which was constant at 150 mL per day without decrease. This was thought to be caused by bile leakage. On d 32 after surgery, a fistulogram obtained from the drainage tube demonstrated a conduit between the anterior bile duct and fistula (Figure 2A). Endoscopic retrograde cholangiography performed on d 39 after surgery demonstrated obstruction of the anterior bile duct (Figure 2B). Taken in sum, these examinations suggested a hole in the distal part of the anterior bile duct with its proximal obstruction. We tried to insert a biliary drainage tube into the anterior branch by percutaneous and endoscopic procedures to decrease the intraductal pressure and close the fistula, but failed. On d 46 after first surgery, a retrograde transhepatic biliary drainage (RTBD) tube was inserted into the anterior bile duct under open surgery. We waited to close the fistula in the outpatient follow-up, however a contrast study of the RTBD taken 7 mo post-surgery showed that the fistula remained patent. Because external biliary drainage continued despite prolonged conservative management, we decided to perform ethanol ablation of the isolated bile duct.

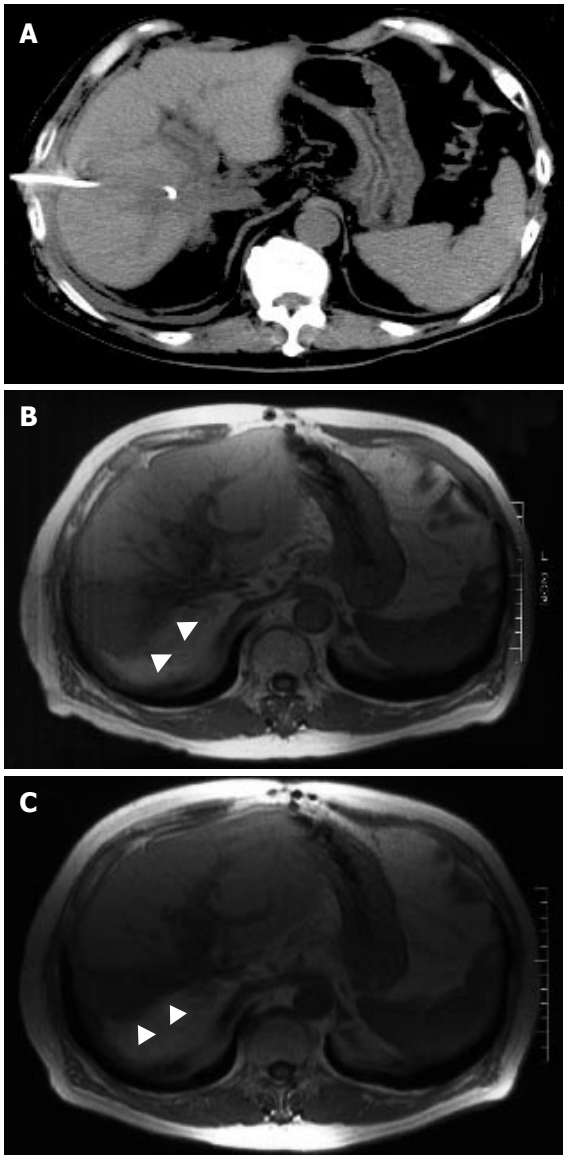


Figure 3 Follow-up computed tomography taken before ethanol injection (**A**), magnetic resonance imaging 3 mo (**B**) and 15 mo (**C**) after injection. Arrows indicate the atrophy of the ethanol-injected anterior segment.

A 5 Fr balloon occlusion catheter was placed in the

anterior bile duct through an RTBD tube. After the balloon was inflated to prevent leakage of the injected ethanol from the bile duct to the fistula, 4.0 mL pure ethanol was injected for ten minutes. Ethanol injection over 4.0 mL caused upper abdominal discomfort, so we decided that 4.0 mL was the appropriate volume. The ethanol injection was repeated five times a week. The volume of bile drainage was diminished to less than 100 mL after five injections, and the injections were continued thereafter. On the 23rd attempt, the volume of bile juice dropped to below 10 mL per day, and then the RTBD was clamped and removed two days later. Total bilirubin concentration of the drainage was also decreased from 61.2 mg/dL pre-injection to 6.9 mg/dL on the 11th attempt, and to 3.9 mg/dL on the 20th attempt. After RTBD removal, the patient had no complaints or symptoms. Compared with computed tomography taken before ethanol injection (Figure 3A), magnetic resonance imaging demonstrated atrophy of the ethanol-injected anterior segment without liver abscess formation (Figure 3B-C).

DISCUSSION

This report indicates that ethanol ablation may be effective in unfortunate cases of refractory biliary leakage after hepatectomy. However, this method can be performed in only biliary fistula without communication with the bile duct of the other remaining liver, because ethanol affects the remaining bile duct and causes irreversible damage. From this point of view, the reported case is thought to be one particularly suitable for ethanol biliary ablation.

Ethanol has been generally used as an injected agent percutaneously or angiographically for the treatment of various tumors because of its highly destructive properties, which lead to cell death by causing cell membrane lysis and protein denaturation[6]. Percutaneous transhepatic ethanol injection to the biliary tract has been reported. Controversy has surrounded chemical ablation of the gallbladder as an alternative to cholecystectomy for over a decade[7-11]. Recently, selective intrahepatic biliary ethanol injection as an alternative to preoperative portal vein embolization[12] or postoperative bile leakage[13] has been introduced. Kyokane *et al* reported that selective intrahepatic biliary ethanol injection destroyed the biliary epithelium, permeated parenchyma, induced hepatocyte denegeration, and resulted in compensatory hypertrophy of the non-injective hepatic lobe in an animal study. Compared with portal embolization, biliary ethanol injection was reported not to cause such severe side effects. In biliary ethanol injection, the hepatocyte denegeration with preservation of hepatic blood supply may prevent clinical liver necrosis or abscess[12]. Li *et al*[14] also demonstrated that biliary injection using phenol plus cyanoacrylate could achieve the effect of chemical hepatectomy.

Although this patient complained of abdominal pain during excessive ethanol ablation, this symptom was promptly relieved when the injection was stopped and the ethanol was removed from the bile duct via an RTBD tube. We were able to find the optimal injected ethanol dose through the patient's complaint and fistulography, after which the patient did not complain of abdominal pain. In this method, the decision on the optimal injected dose may be important to prevent side effects[15].

Unfortunately, biliary leakage is sometimes encountered, and it can be complicated when it does occur[3,5,16]. Ethanol ablation of the bile duct provides a management option in cases in which conservative treatment has failed and a surgical approach is relatively difficult.

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CASE REPORT

Synchronous anorectal melanoma

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Abstract

Anorectal melanoma is a very rare tumor with poor prognosis. Rectal bleeding is the most frequent symptom and surgical treatment ranges from local excision to radical abdominoperineal resection. We report a case of a 75-years-old male patient who presented with a history of recurrent rectal bleeding, and whose histopathological diagnosis was melanoma. Macroscopically, we found two distinct tumors in anorectal region, 0.5 cm and 1.5 cm from dentate line. The first one was pedunculated, on a thin stalk, measuring 1 cm in greatest diameter, and the second one was sessile and nodular measuring up to 2.8 cm in largest diameter. Microscopic examination and immunohistochemical analysis of both tumors confirmed the diagnosis of melanoma. This case represents multiple synchronous primary melanoma of the anorectal region, with a possibility that one of the lesions is primary melanoma and the second one is a satellite lesion.

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Key words: Melanoma; Anorectal region; Satellite lesions; Synchronous melanomas

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INTRODUCTION

Anorectal melanoma is a rare condition accounting for 0.2%-3% of all melanoma cases, and 0.1%-4.6% of all malignant tumors of the rectum and anus[1,2,3]. Melanoma is defined as "rectal" when it occurs in the rectum above the anorectal junction[4], while anal canal is below anorectal line. Rectal bleeding was the most common reported symptom, and sometimes the importance of the bleeding is underestimated as it was assumed to be a sign of hemorrhoid disease causing a delay in making the correct diagnosis and starting treatment[4,5]. Surgical treatment of this tumor ranges from the radical abdominoperineal resection with bilateral inguinal lymphadenectomy, to the conservative local excision alone[4]. Long-term survival is very low; approximately 6.7% to 12% of patients were reported to be free of disease five years after their operations[6,7]. Most recent studies did not find correlation between tumor necrosis and blood vessel invasion and survival rate, while correlation of survival and tumor thickness is controversial. In the most studies, tumors evaluated by flow cytometry that showed aneuploidy correlated with poorer prognosis. S-phase fraction higher than 15% also correlated with a worse prognosis[4,5].

CASE REPORT

A 75-year-old male patient presented with a history of recurrent rectal bleeding which had become more frequent and severe during the previous year. Colonoscopy revealed only one polipoid and fragile mass in the rectum, and punch biopsy showed pigmented melanoma. Computed tomography (CT) scan and ultrasound did not find enlargement of regional lymph nodes.

Pathologic examination of the received specimen revealed two distinct tumorous masses; the first one that was pedunculated, on a thin stalk, 0.5 cm above dentate line, measuring 1 cm in greatest diameter. The second one was sessile and nodular, 1.5 cm above dentate line, measuring up to 2.8 cm. Both tumors were bluish brown on cut sections (Figure 1A).

After surgery, all specimens were formalin fixed, paraffin embedded, cut at 5 μm and routinely stained with hematoxylin and eosin. Deparaffinisation and immunohistochemical staining was performed following Microwave Streptavidin ImmunoPeroxidase (MSIP) protocol on DAKO TechMate™ Horizon automated immunostainer. We used primary antibodies to HMB-45 and S-100 (purchased from DAKO, Copenhagen, Denmark). Microscopically, both tumors were composed of atypical spindle cells with prominent nucleoli and highly pigmented. First tumor, the smaller one, was covered with squamous epithelium, partially ulcerated, infiltrating mucosa and submucosa but without extension to the

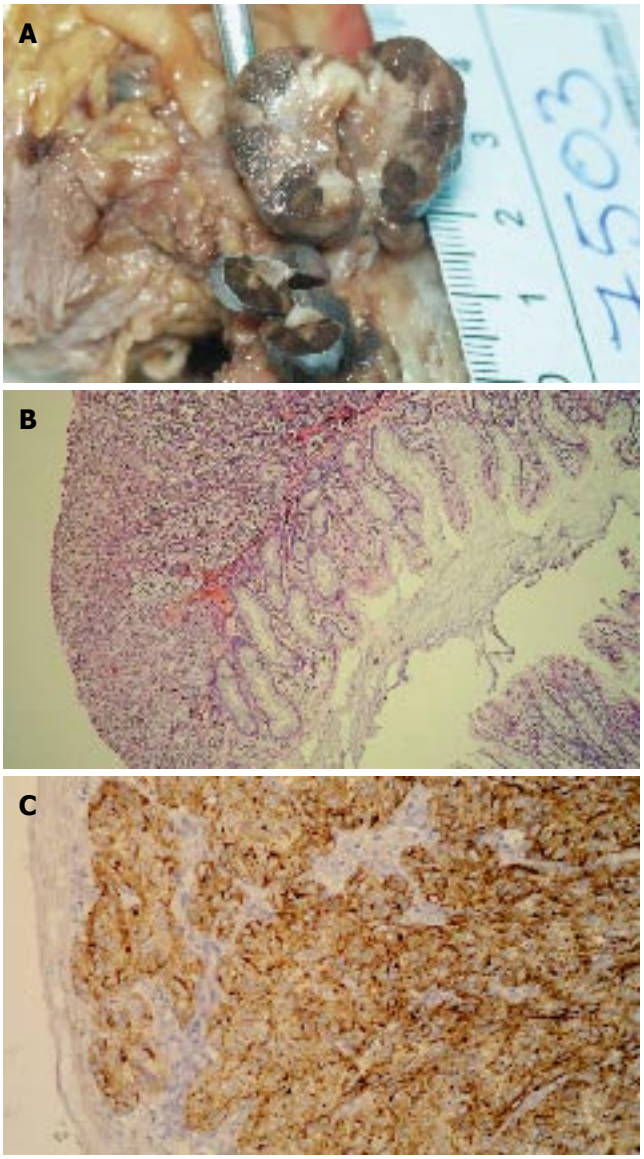


Figure 1 **A**: Macroscopical appearance of anorectal region with two melanomas; **B**: Photomicrograph of rectal melanoma covered with columnar epithelium (HE, original magnification x 100); **C**: Photomicrograph of anal melanoma covered with squamous epithelium showing strongly positive immunostaining for HMB-45 (original magnification x 200).

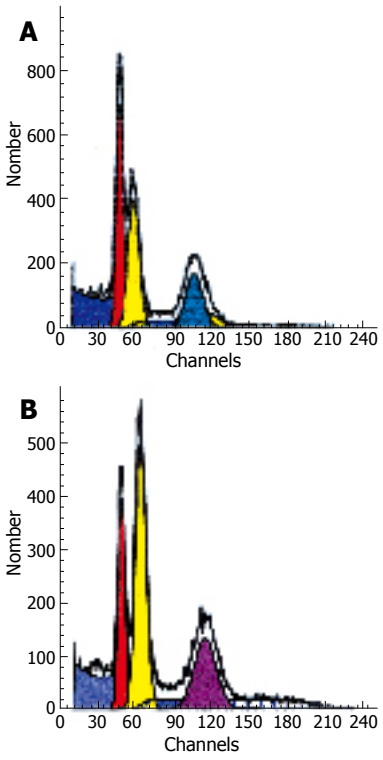


Figure 2 DNA histograms. **A**: Smaller tumor with two aneuploid peaks: left peak-hypodiploid population with DNA index of 1.22; right peak-hyperdiploid population with DNA index of 2.22. Middle peak-the diploid population of non tumoral cells; **B**: Larger tumor with two aneuploid peaks: left peak-hypodiploid population with DNA index of 1.32; right peak-hyperdiploid population with DNA index of 2.33. Middle peak-the diploid population of non tumoral cells.

muscle layer. Mitotic rate was 10/10 HPF. The larger tumor was covered with partially ulcerated columnar epithelial cells, without muscle layer infiltration (Figure 1B). Mitotic rate was 13/10 HPF. Tumor thickness, measured by computer program, was 5.75 mm for the smaller tumor, and 9.11 mm for the larger one. Both tumors showed immunohistochemically positive reaction for HMB-45 and S-100 (Figure 1C).

Paraffin embedded specimens were processed for the flow cytometry. Cellular DNA content and proliferative activity were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using excitation wavelength at *A*488 nm and 15-mW argon ion laser. DNA histograms were generated on at least 20 000 nuclear events with a Mod-FitLT V2.0 (Verity Software House Inc.,Topshame, ME and Becton Dickinson).

DNA flow cytometry analysis showed two aneuploid peaks (hypodiploid and hyperdiploid populations) in both tumors, but with different DNA indexes and S-phase fractions (Figure 2A, 2B). S-phase fraction for the polipoid tumor, covered with squamous epithelium was 11.84%, and for the nodular one, covered with columnar epithelium was 26.82%.

DISCUSSION

Anorectal melanoma is a very rare neoplasm, and in the reviewed literature we did not find any similar case of anorectal melanoma, either with satellite lesion or with multiple melanomas in anorectal region. We found only one reported case of metachronous melanoma in the upper digestive system, involving esophagus and stomach[8]. The case presented in this paper is specific by reporting two, synchronous melanomas, of which one of the tumors was found in anal canal, and it was partially covered by ulcerated squamous epithelium, while the other one was in rectum, covered with partially ulcerated columnar epithelium. Between described tumors there was an area of normal rectal mucosa without tumor involvement. For almost 30 years patient had recurrent bleeding because of hemorrhoid disease, which probably caused delay in making a correct diagnosis. Although some authors did not find difference in overall survival between patients treated with local excision and those with abdominoperineal resection[2,9], the study from the Memorial Sloan Kettering Cancer Center by *Brady et al.* suggested abdominoperineal resection as a better approach for patients without advanced disease, because patients treated with abdominoperineal resection were more commonly associated with better long-term survival than those treated with other procedures[4,6]. After physical, CT scan and ultrasound examination which did

not find any signs of metastatic disease, patient was treated by radical abdominoperineal resection.

The most important doubt in this case is the nature of both lesions. Are they both primary anorectal melanoma or is one of the two tumor primary, and which one, and the second one is probably a satellite lesion?

In skin melanomas, satellite lesions are defined as tumor nests or nodules within 2 cm of the primary tumor, while in-transit metastasis involves skin or subcutaneous tissue more than 2 cm from the primary tumor, but not beyond the regional lymph nodes. The significance of their presence is seen in survival studies, where lymph node metastasis is one of the most important predictor factors of survival in melanoma patients, and presence and number of involved lymph nodes is more important than actual gross dimension of the involved lymph node. In new melanoma staging system, patients with in-transit metastases or satellite lesions without lymph node disease have a similar prognosis to those with positive lymph node metastases. In the revised staging system, these patients are now upstaged as having N2c disease compared with the 1997 staging system in which these patients were considered as having T4 disease[10].

In this case, it is likely that one of the lesions is primary tumor and the second one is the satellite lesion. Concerning colonoscopy, which was performed few months before surgery, and which found only one polipoid tumor, it is possible that the smaller tumor is primary, and the second one is a satellite lesion. Hypothesis that one of the tumors is a satellite lesion is also supported with histological findings, because both tumors are composed of the same histological type. However, the surface of both tumors is ulcerated, making it very difficult to establish connection of the tumor cells with the surface, and concerning different S-fases on DNA flow cytometry there is still a possibility that this is the case of a multiple synchronous primary melanoma in anal canal and rectum.

Importance of distinguishing exact nature of the found lesions, if we could apply the same rules as for staging cutaneous melanoma, is better staging and predicting prognosis.

This case represents the multiple synchronous primary melanoma of the anorectal region, with a possibility that one of the lesions is primary melanoma and the second one is a satellite lession.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology
Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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4–5 May 2006
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EDITORIAL

Role of microbubble ultrasound contrast agents in the non-invasive assessment of chronic hepatitis C-related liver disease

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Abstract

Patients who are chronically infected with the hepatitis C virus often develop chronic liver disease and assessment of the severity of liver injury is required prior to considering viral eradication therapy. This article examines the various assessment methods currently available from gold standard liver biopsy to serological markers and imaging. Ultrasound is one of the most widely used imaging modalities in clinical practice and is already a first-line diagnostic tool for liver disease. Microbubble ultrasound contrast agents allow higher resolution images to be obtained and functional assessments of microvascular change to be carried out. The role of these agents in quantifying the state of hepatic injury is discussed as a viable method of determining the stage and grade of liver disease in patients with hepatitis C. Although currently confined to specialist centres, the availability of microbubble contrast-enhanced ultrasound will inevitably increase in the clinical setting.

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Key words: Hepatitis C; Microbubble contrast agents; Ultrasound

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INTRODUCTION

It is estimated that 170 to 200 million people (around 3% of the global population) are chronically infected with the hepatitis C virus (HCV), while around 4 million people are newly infected each year[1]. There is a great degree of variation in prevalence, ranging from less than 1% in the United Kingdom to more than 20% in Egypt. In the developed world, the relatively low prevalence is mostly the result of intravenous drug abuse, while a World Health Organisation (WHO) anti-schistosomal vaccination programme in Egypt in the 1970s and other vaccination programmes in Mongolia and Bolivia have led to much higher rates in those countries[2]. Between 55% and 85% of those infected go on to develop chronic liver disease and even then, many remain asymptomatic, while the rest suffer primarily from chronic fatigue[3]. Around 20% of patients with fibrosis develop cirrhosis and 5% of these are susceptible to developing hepatocellular cancer each year (Figure 1)[4]. A number of factors have been linked to the progression of chronic hepatitis, with some being implicated not only in advancing the progression, but also worsening the overall prognosis[5]. These factors include age at infection greater than 55 years, male sex, excessive alcohol consumption, a long history of cigarette smoking and the presence of hepatic steatosis, obesity and diabetes mellitus (the metabolic syndrome)[5].

ASSESSING CHRONIC HEPATITIS C-RELATED LIVER DISEASE

An assessment of the severity of hepatic involvement is obviously required prior to considering antiviral treatment regimens with interferon and ribavirin in patients with hepatitis C. In addition, owing to the variable disease progression, the potentially life-threatening complications of end-stage liver disease and the development of hepatocellular carcinoma, it is also imperative to monitor hepatitis C-infected patients with established cirrhosis.

LIVER BIOPSY

For many years, the gold standard for assessing chronic

Table 1 Commonly available serological markers for assessing liver injury[8]

Group	Marker/panel	Comments
1	Prothrombin index	Highest diagnostic accuracy (cirrhosis) 86%
1	PGAA	Diagnostic accuracy 80%
1	AST/ALT ratio	Increase strongly correlated with decrease in hepatic function AST/ALT > 1 can diagnose cirrhosis with sensitivity of 77.8% and specificity 96.9%, but misclassified 7% as having cirrhosis and failed to diagnose 11%
1	FibroTest	Sensitivity 75%, specificity 85%
1	APRI	Predicts fibrosis in 51% patients Predicts cirrhosis in 81% patients
2	Hyaluronic acid (HA)	Only group 2 marker to show encouraging results, with a positive predictive value similar to Child-Pugh

hepatitis has been histological examination of liver biopsy material. However, this invasive procedure is not without risks with a small, but defined mortality rate and it has a significant error rate, sampling less than $1/50\,000^{th}$ of the liver volume even in cases of diffuse disease[6-8]. As a result of the problems associated with biopsy, there has been a steady drive in recent times to find an effective non-invasive method of evaluating liver injury, which has led to major developments in both serological markers of disease and imaging.

SEROLOGICAL MARKERS

An ideal serological marker for assessing liver injury should be liver-specific, easy to use and allow measurement of the stage of disease using the degree of fibrosis, the activity of matrix deposition and the activity of matrix removal[8]. These can be broadly classified into two groups. The first (Group 1 in Table 1) are those which are indirect markers of fibrosis, reflecting alterations in hepatic function, and include PGAA {prothrombin index, gamma glutamyl transpeptidase (γGT), apolipoprotein A_1 and α_2-macroglobulin}[8], AST/ALT (aspartate aminotransferase and alanine aminotransferase)[9], FibroTest (α_2-macroglobulin, α_2-globulin, γ-globulin, apolipoprotein A_1, γGT and total bilirubin)[10] and APRI (AST to platelet ratio index)[11]. The second (Group 2 in Table 1) are those which are direct markers of fibrosis, reflecting extracellular matrix metabolism (Group 2 in Table 1)[8]. The current issue is that many of these proposed panels have not been widely validated around the world and often remain championed largely by the research groups that initially proposed them. Currently, none fulfils all the criteria for an ideal serological marker and none has yet achieved the levels of sensitivity and specificity seen with liver biopsy.

IMAGING

Of all the proposed non-invasive alternatives to liver biopsy, imaging has shown some promise. Using routine grey-scale ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI), it has been shown that a small right hepatic lobe, with relatively enlarged left and caudate lobes, are reliable markers of cirrhosis, having a sensitivity of 84%, specificity of 100% and diagnostic accuracy of 94%. These figures are well beyond any achieved by the many serological markers[8].

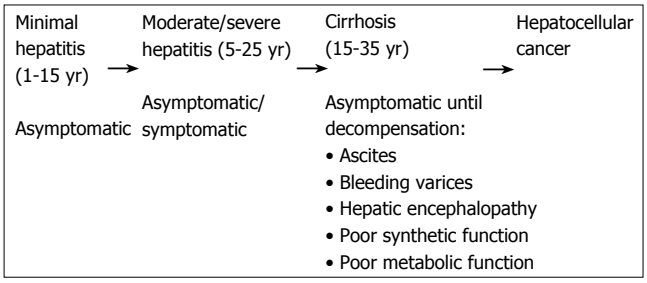


Figure 1 Time scale for progression of hepatitis C-related liver disease[3,4].

ULTRASOUND IMAGING OF THE LIVER

Ultrasound is one of the most widely used imaging modalities in clinical practice. The major attraction is that it is already the first line of imaging in the diagnosis of liver disease and is of particular interest, as it has been shown to be complementary in patients with equivocal biopsy results or where biopsy is contraindicated, but cirrhosis is suspected[8]. It is also used to screen patients with chronic hepatitis for hepatocellular cancer, enabling detection and characterisation of tumors[12]. In patients with cirrhosis, it is possible to demonstrate nodularity of the liver surface and parenchyma and sometimes atrophy of the right lobe[13], but for the most part, standard grey-scale imaging does not render much information on the extent of fibrosis in pre-cirrhotic disease. The most accurate diagnoses which can be made on grey-scale imaging are those of hepatic steatosis, demonstrated by parenchymal hyperechogenicity (Figure 2), and the presence of a nodular surface in cirrhosis (Figure 3). The need to discriminate pre-cirrhotic liver disease non-invasively has driven the development of additional ultrasound techniques, including elastography and a group of microbubble ultrasound contrast agents, in order to achieve better resolution images and allow a degree of hepatic functional assessment.

Transient elastography

Transient elastography has recently been used to characterize the stiffness of liver tissue *in vivo*, which has been correlated with the histological grade of fibrosis in hepatitis C[14]. A probe vibrating at a predetermined amplitude and frequency is placed against skin overlying the liver. The resultant shear wave is propagated through tissue at a velocity dependent on its stiffness. The velocity can be measured by one-dimensional ultrasound along the axis of the transmitted wave. Although correlation is good overall, there is significant overlap of stiffness as measured by elastography between the different stages of fibrosis. Elastography has been used in conjunction with the serological markers, FibroTest (Table 1 for details) and the aspartate aminotransferase (AST) to platelet ratio (APRI) to evaluate

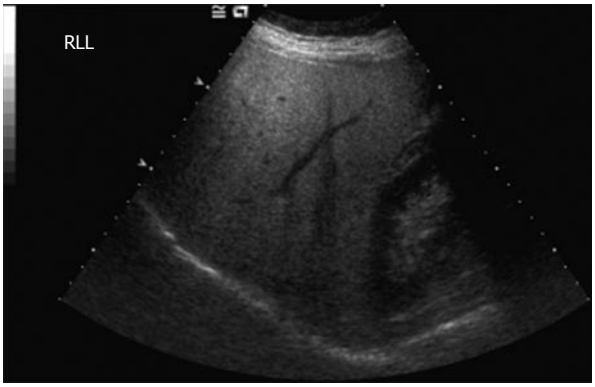


Figure 2 Ultrasound image of hepatic steatosis, showing increased echotexture of the liver parenchyma in comparison to the right kidney.

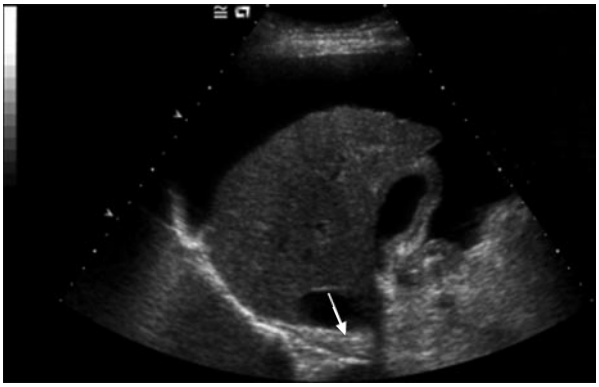


Figure 3 Ultrasound image of a typical cirrhotic liver with a shrunken right lobe, a nodular surface (white arrow), surrounding ascites and a heterogeneous echotexture.

liver fibrosis. There was correlation with histology, but significant overlap of elastography values between stages of fibrosis[15,16]. Further larger scale studies are required using this methodology.

Ultrasound contrast imaging using microbubbles

In the late 1960s, a group of echocardiographers discovered, quite by accident, that the microscopic bubbles of air which enter the circulation during intravenous injections produce strong echoes, where normally none is seen[17,18]. This discovery led to the development of the first type of microbubble contrast agents for ultrasound, which became available in the mid-1990s. Microbubbles are tiny, gas-filled bubbles with a shell (made from denatured albumin, phospholipids, surfactant or cyanoacrylate) which measure less than 10 μm in diameter and are small enough to pass through the cardiopulmonary circulation and also cross capillary beds[19].

In simple terms, microbubbles are echo-enhancers, which enhance both grey-scale and Doppler ultrasound signals by up to 25 dB, giving an increase of more than 300 folds[20]. The first microbubble agent to be licensed for use in the United Kingdom was Levovist® in 1997. Although it exhibited a degree of liver specificity, it had a short half-life and the bubbles were mostly destroyed by liver, meaning that only one pass through the liver was possible. The introduction of SonoVue®, a third-generation microbubble agent, in 2001 allowed real-time contrast-enhanced imaging and multiple passes through the liver.

Safety of microbubbles

As with all pharmaceutical agents, microbubble agents have to pass rigorous safety tests in order for them to be licensed. Extensive trials have established an excellent overall safety record with few significant adverse effects[20]. However, there are some concerns that disruption of their outer shells produces local bioeffects such as sonoporation (subcellular membrane damage) and cell lysis at diagnostic frequencies and that these effects are enhanced by perfluorocarbon gases. Despite this, no effects of these processes have been observed in humans[20]. Concern about administering volumes of gas into the blood stream has been shown not to be an issue as the amount given is under 200 μL, too small to exhibit an effect[21]. Overall, it has been shown that the safety of microbubbles compares favourably to that of conventional radiographic contrast agents and those used in contrast-enhanced MRI[21].

Applications of microbubbles to the assessment of liver disease

Microbubbles have a multitude of clinical uses, but are best known for imaging the vasculature, contrast-enhanced echocardiography, imaging the liver and functional studies. In these functional studies, following an intravenous bolus injection, their passage can be tracked through a tissue or organ and used to generate "transit time" curves in much the same way as those in nuclear medicine, CT and MRI are produced[20].

One of the major applications of transit times is in the assessment of liver disease and the non-invasive diagnosis of cirrhosis[21]. Severe liver disease leads to characteristic haemodynamic changes affecting hepatic blood flow. The development of a hyperdynamic circulation (increased cardiac output, reduced systemic vascular resistance, altered circulating vasoactive factors-glucagons and prostaglandins), pulmonary arteriovenous shunts, venous collaterals (due to increased portal pressure), intrahepatic shunts (between hepatic artery, portal vein and hepatic veins), and arterialisation of the liver capillary beds, results in the early arrival of a bolus of microbubbles injected peripherally (Figure 4). The hepatic vein transit time (HVTT) is the difference between the hepatic artery and the hepatic vein arrival times.

Many of the published studies[22-24] assessing HVTT in patients with chronic liver disease have employed measurements using the spectral Doppler technique, where the arrival time is calculated as a rise in Doppler intensity 10% above baseline. This requires specialized software to evaluate the HVTT, but is relatively straight-forward, as shown in Figure 5. The figure demonstrates a normal hepatic vein transit time (43 s) in a patient without significant liver disease (upper graph, Figure 5) and a comparative early HVTT in a patient with cirrhosis (14 s) (lower graph, Figure 5). There is a much shorter HVTT in the patient with cirrhosis. It is important to note that 20 s of baseline is collected before the injection of the microbubble contrast agent. The continuous horizontal line denotes a Doppler intensity 10% above baseline and hence the HVTT is taken

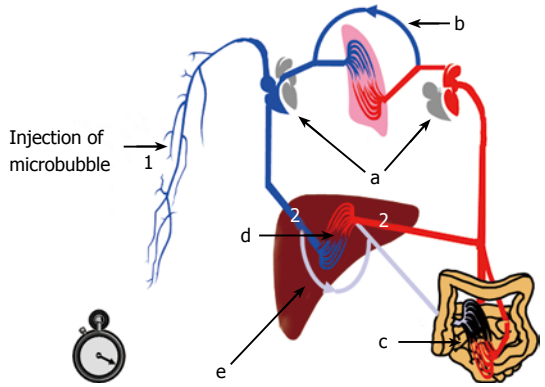


Figure 4 Characteristic haemodynamic changes seen in severe chronic liver disease which lead to the early arrival of microbubbles in the liver. Time is measured from injection (1) to arrival in the liver (2).

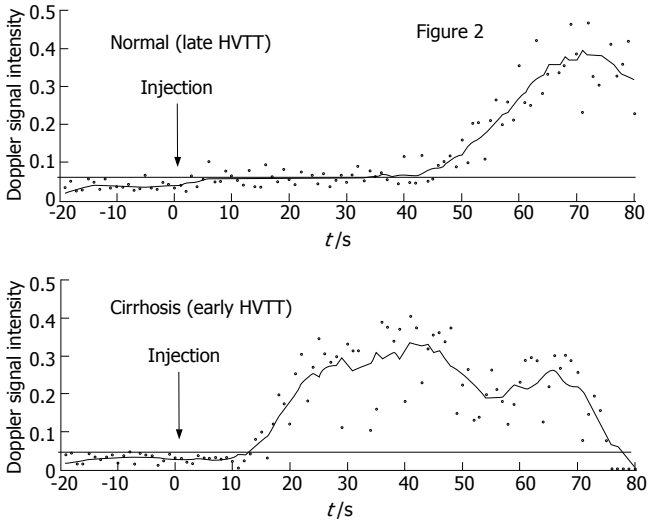


Figure 5 Hepatic vascular transit times (HVTT) following a bolus injection of a microbubble contrast agent, measured in a volunteer without significant liver disease (upper graph) and a patient with cirrhosis (lower graph). There is a much shorter HVTT in the patient with cirrhosis. Adapted from Lim *et al*[23].

as the intersection of this line with that of the Doppler intensity trace (Figure 5).

The development of newer more stable microbubbles, such as SonoVue® (Bracco, Milan, Italy) and improvement in ultrasound capabilities have allowed 'real-time' evaluation of the transit times which can be readily visualized arriving in the hepatic arteries and veins (Figures 5 and 6) without the need for 'off-line' processing.

A typical, simplified transit time assessment would now involve the injection of a bolus of the microbubble agent into an antecubital fossa vein and measurement of the time taken for the contrast agent to be seen in the liver. The first flash of microbubbles seen in the hepatic arteries is recorded as the hepatic artery arrival time. A continuous stream of microbubbles in the hepatic vein is recorded as the hepatic vein arrival time. The hepatic vascular transit time (HVTT) is thus the difference between the hepatic artery and the hepatic vein arrival times.

It has been shown that these transit times can be linked to progression in the Child-Pugh score, becoming shorter as the liver disease worsens[22]. Pre-cirrhotic disease due to hepatitis C can now be classified into mild ($\geqslant$ 28 s) or moderate to severe ($\geqslant$ 22 s, $<$ 28 s) according to transit times[20]. A recent study has shown that a transit time of $\geqslant$ 21 s is 100% sensitive and 96% specific for cirrhosis, making it a viable alternative when liver biopsy is contraindicated. It is also perhaps a method of monitoring disease progression in response to treatment[23]. Figure 6 is adapted from a recent study by Lim *et al*[23] which shows a progressive shortening of the HVTT with increasing severity of liver disease in a cohort of patients with HCV-related liver disease. There is clear separation between the groups (categorisation of patients was carried out according to the Ishak histological scoring system[25]). Importantly, there is a clear separation, not only between the patients with moderate hepatitis from those with cirrhosis, but also in the pre-cirrhotic groups, where there is good separation of patients with mild hepatitis from those with moderate hepatitis[23]. This may be clinically relevant and demonstrates for the first time the possibility that ultrasound techniques can actually monitor the severity of pre-cirrhotic disease non-invasively.

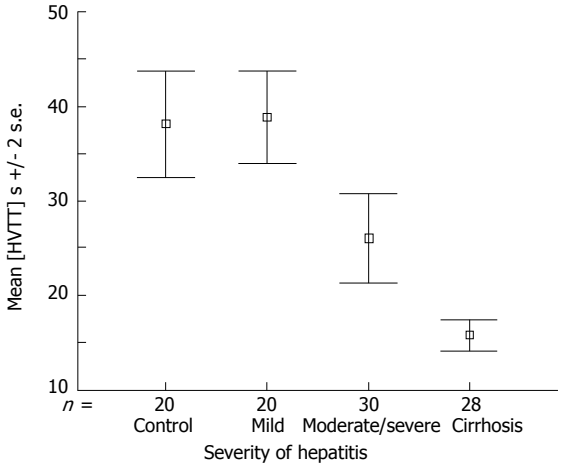


Figure 6 Graph showing hepatic vascular transit times (HVTT) in patients with mild hepatitis, moderate to severe hepatitis and established cirrhosis. There is progressive shortening of the HVTT with increasing severity of liver disease. Adapted from Lim *et al*[23].

Newer microbubble contrast agents

Levovist is at present the most commonly used microbubble agent, but there are several newer agents, such as SonoVue® (Bracco, Milan, Italy) or Definity® (Bristol-Myers-Squibb, New York, USA), which have different chemical properties to Levovist®, but also provide enhanced Doppler intensity when they arrive in the hepatic veins. It remains to be shown whether the HVTT for these agents are similar and whether they can be used interchangeably. A benefit of using these newer agents is that their arrival within a hepatic vein can be observed in 'real-time', using low acoustic power two-dimensional harmonic scanning modes. The main benefit of using these modes is that it would not require computer-aided post-processing of the data to calculate the HVTT, thus making it accessible to any department with a modern ultrasound machine.

CONCLUSION

There are clearly a number of very different methods currently available for the monitoring of hepatitis C-related liver disease. Although liver biopsy remains the gold standard, it is a highly invasive procedure that is not without risk and is open to sampling error, particularly in disease where there is patchy involvement. There is a continual drive towards minimally or non-invasive testing, and therefore, alternatives to liver biopsy are being actively sought.

The use of serological markers remains somewhat confused, with prothrombin index having the highest diagnostic accuracy, despite many other panels being suggested. Further research by independent teams is required to understand exactly what role these markers can perform.

Developments in imaging technology in the past decade have yielded sensitivities and specificities similar to that of liver biopsy for the first time. Ultrasound continues to be the primary imaging modality of choice when investigating liver disease. Although currently limited to a few specialist centers, microbubble contrast agents will inevitably become much more widely used. Hepatic vein transit time measurement using an ultrasound microbubble agent is a simple, non-invasive test which can be used to stage and grade pre-cirrhotic liver disease with clear separation between mild hepatitis and cirrhosis, and statistically significant differences between these two groups and moderate to severe hepatitis. The response to treatment using hepatic vein transit times needs to be assessed in future longitudinal studies, but the methodology is potentially translatable as an efficient, quick and discriminative test for the assessment of disease severity in hepatitis C to any ultrasound department anywhere in the world.

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Calcium signaling in cholangiocytes

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Abstract

Cytosolic Ca^{2+} is an important second messenger in virtually every type of cell. Moreover, Ca^{2+} generally regulates multiple activities within individual cells. This article reviews the cellular machinery that is responsible for Ca^{2+} signaling in cholangiocytes. In addition, two Ca^{2+}-mediated events in cholangiocytes are discussed: bicarbonate secretion and apoptosis. Finally, emerging evidence is reviewed that Ca^{2+} signaling is involved in the pathogenesis of diseases affecting the biliary tree and that Ca^{2+} signaling pathways can be manipulated to therapeutic advantage in the treatment of cholestatic disorders.

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Key words: Ca^{2+}; Cholangiocyte; Inositol trisphosphate; Bile secretion; Cholestasis; Apoptosis

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INTRODUCTION

Cytosolic Ca^{2+} is an important second messenger in virtually every type of cell. Ca^{2+} regulates a wide range of cell functions, including contraction, gene transcription, cell growth, differentiation, apoptosis, membrane fusion, ion channel activation, and secretion[1]. Moreover, Ca^{2+} generally regulates multiple activities within individual cells. Temporal Ca^{2+} signaling patterns, such as Ca^{2+} oscillations, and spatial signaling patterns, such as Ca^{2+} gradients and waves, permit Ca^{2+} to exhibit this differential regulation of cell function. Therefore, the manner in which cells organize their Ca^{2+} signals is critical for the types of signals they can produce, and thus is critical for the ways in which Ca^{2+} can regulate cell function. There are certain common mechanisms for generating Ca^{2+} signals among cell types, but each cell type has distinct features of signaling machinery as well. Here we will review the machinery that is responsible for Ca^{2+} signaling in cholangiocytes. We also will discuss in detail two Ca^{2+}-mediated events in cholangiocytes, bicarbonate secretion and apoptosis. Finally, we will review emerging evidence that Ca^{2+} signaling plays an important role in the pathogenesis of diseases affecting the biliary tree and in the treatment of cholestatic disorders.

Ca^{2+} SIGNALING MACHINERY IN CHOLANGIOCYTES

Cytosolic Ca^{2+} signals originate from two general sources, release from intracellular Ca^{2+} stores and influx across the plasma membrane. The initial component of Ca^{2+} signals in cholangiocytes and other epithelia is due entirely to Ca^{2+} release from intracellular stores, since this component is unchanged in Ca^{2+}-free medium[2,3]. In contrast, the role of extracellular Ca^{2+} is to maintain cytosolic signals over longer time intervals and to replenish intracellular stores. This review will therefore focus on intracellular Ca^{2+} release mechanisms. There are two intracellular Ca^{2+} release channels that are principally responsible for Ca^{2+} release in nearly every cell type, the ryanodine receptor (RyR) and the inositol 1,4,5-trisphosphate (InsP3) receptor (InsP3R)[1]. The RyR is most heavily expressed in excitable cells such as myocytes and to a lesser extent in neurons. The RyR also is expressed in some nonexcitable cells, including certain polarized epithelia, such as pancreatic acinar cells[4]. The RyR is localized to sarcoplasmic or endoplasmic reticulum (ER), where most intracellular Ca^{2+} is stored. Activation of the receptor allows Ca^{2+} to be released from the ER into the cytosol. The RyR can be activated in several different ways, including through direct coupling with certain plasma membrane Ca^{2+} channels, *via* stimulation with the second messenger cyclic ADP-ribose, or by stimulation with Ca^{2+} itself (Ca^{2+}-induced Ca^{2+} release). Many epithelia, including cholangiocytes[5] as well as hepatocytes[6], do not express the RyR.

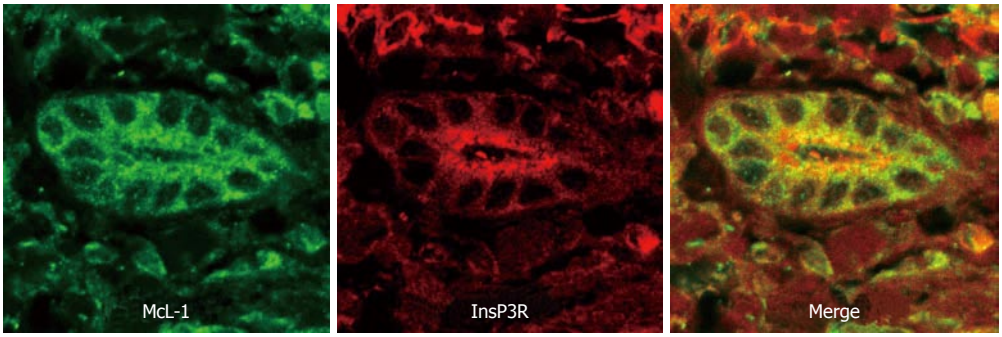


Figure 1 Distribution of the InsP3 receptor and Mcl-1 in human bile ducts. This confocal immunofluorescence image was obtained from a paraffin-embedded biopsy specimen from a normal liver. The specimen was double-labeled with antibodies against Mcl-1 (green) and the type III InsP3R (red). Note that the Mcl-1 is distributed diffusely throughout the cytosol, while the InsP3R is most concentrated in the apical region.

The InsP3R is expressed in virtually every cell type, is the predominant Ca^{2+} release channel in epithelia, and is the only intracellular Ca^{2+} release channel in cholangiocytes[5]. Like the RyR, the InsP3R is expressed in the ER. The endogenous ligand for the InsP3R is InsP3, which is formed from phospholipase C (PLC)-induced hydrolysis of phosphatidyl inositol bisphosphate (PIP2)[7]. This biochemical pathway can be activated by stimulation of either G protein-coupled receptors or receptor tyrosine kinases[1]. Therefore, stimulation of cholangiocytes with certain hormones or growth factors results in InsP3-mediated increases in cytosolic Ca^{2+}. Hormone-induced Ca^{2+} signaling is mediated entirely by InsP3 in cholangiocytes, since Ca^{2+} signaling is blocked by microinjection of the InsP3R inhibitor heparin[2]. There are three InsP3R isoforms, each of which has distinct biophysical properties[8,9]. These properties result in different effects on Ca^{2+} signaling. For example, in DT40 cells engineered to express only a single InsP3R isoform, activation of the type Ⅰ InsP3R results in transient Ca^{2+} oscillations, while the type Ⅱ InsP3R induces sustained oscillations, and the type Ⅲ isoform results in a single, transient increase in Ca^{2+}[10]. Similarly, in CHO cells in which expression of each one of the three isoforms has been silenced, ATP-induced Ca^{2+} signals are preferentially converted from oscillations to either sustained or transient increases in Ca^{2+}[11]. Many cell types express multiple InsP3R isoforms, and some cells[12,13], including cholangiocytes[5], express all three isoforms. Even among cell types that express all three isoforms, there is considerable variability in terms of how much of each isoform is expressed, and in terms of the subcellular region in which each isoform is expressed. In cholangiocytes, the type Ⅲ InsP3R accounts for about 80% of InsP3Rs, while the types Ⅰ and Ⅱ isoforms each account for about 10%. Moreover, the type Ⅲ InsP3R is most concentrated in the apical region (Figure 1), while the other two isoforms are not distributed in a polarized fashion[5]. Hormone-induced Ca^{2+} signals in cholangiocytes begin as apical-to-basal Ca^{2+} waves[5], similar to what is observed in other polarized epithelia[6,14]. In cholangiocytes, this polarized Ca^{2+} signaling pattern is likely due to the apical concentration of the type Ⅲ InsP3R, although it is not clear if this is because the receptor has properties that permit it to trigger Ca^{2+} waves[8], or simply because of the increased InsP3R concentration in the apical region[15]. Ca^{2+} signals also can spread among neighboring cholangiocytes. This effect is mediated by gap junctions in most epithelia[16], including cholangiocytes[17]. The predominant gap junction protein in cholangiocytes is connexin 43, although connexin 32 is expressed in these cells to a lesser extent[17]. Permeability of cholangiocyte gap junctions is decreased by protein kinases A and C[17], which provides additional ways in which Ca^{2+} signals can be modulated in intact bile ducts.

INTERCELLULAR MESSENGERS THAT ACT THROUGH Ca^{2+}

Several intercellular messengers have been identified that increase cytosolic Ca^{2+} in cholangiocytes. Each messenger molecule interacts with specific G protein-coupled receptors that increase Ca^{2+} *via* activation of PLC and formation of InsP3. Acetylcholine (ACh) increases Ca^{2+} by stimulation of M3 muscarinic receptors, which have been identified on cholangiocytes by RT-PCR, immunoblot, and immunochemistry[18]. These receptors are expressed on the basolateral membrane[2]. ACh-induced increases in Ca^{2+} can have both indirect and direct effects on downstream signaling pathways. ACh-induced increases in Ca^{2+} activate calcineurin, which potentiates increases in cAMP that are induced by secretin[18]. Stimulation of cholangiocytes with ACh can result in a sustained, transient, or oscillatory increase in cytosolic Ca^{2+}[2]. The likelihood that a particular pattern of Ca^{2+} signal will be elicited is not dose-dependent, though. ATP also increases Ca^{2+} in cholangiocytes[2]. Like ACh, ATP can induce a sustained, transient, or oscillatory increase in cytosolic Ca^{2+}. Unlike ACh, lower concentrations of ATP predominantly induce Ca^{2+} oscillations, while higher concentrations induce single (either sustained or transient) increases in Ca^{2+}[2]. RT-PCR evidence suggests that cholangiocytes express P2Y1, P2Y2, P2Y4, P2Y6, and P2X4 nucleotide receptors[19]. Pharmacologic evidence using microperfused bile duct segments isolated form rat liver suggests that each of the four P2Y subtypes are expressed on the apical membrane of cholangiocytes. P2Y receptors also are expressed on the basolateral membrane, but the actions of ATP in that region are attenuated by ecto-nucleotidases expressed at that membrane[19] or by nearby portal fibroblasts[20]. Pharmacological studies suggest that there is little or no functional expression of P2X receptors in cholangiocytes[19]. These findings suggest that the principal route for signaling to cholangocytes through ATP is *via* bile. ATP may be released from cholangiocytes to signal in an autocrine or paracrine fashion[21], and this pathway is important for cell volume regulation[22]. ATP also can be secreted into bile from hepatocytes[21,23], which provides a pathway through which hepatocytes can signal

to cholangiocytes[24]. The manner in which this signaling pathway is regulated has not been established, but certain bile acids appear to activate this pathway in order to stimulate ductular bicarbonate secretion[23].

Cholangiocytes also express receptors for a number of neuropeptides that act through Ca^{2+} signaling. Cholangiocytes express both the serotonin 1a and 1b receptor on their basolateral membrane[25]. Stimulation of this receptor inhibits the bile duct proliferation that occurs following bile duct ligation. This is likely mediated through Ca^{2+} signaling, since receptor stimulation is associated with an increase in InsP3 and the effects on cholangiocyte proliferation are blocked by chelation of cytosolic Ca^{2+}[25]. Work in cholangiocyte cell lines has shown that these cells also express CCK-B gastrin receptors. As with serotonin receptors, stimulation of gastrin receptors inhibits cholangiocyte growth. The effect of gastrin also is blocked by chelation of cytosolic Ca^{2+}[26]. Cholangiocytes express alpha-1 adrenergic receptors, primarily on their basolateral membrane. The alpha-1 adrenergic agonist phenylephrine increases secretin-stimulated ductal secretion, as measured in both intact rat liver and isolated rat bile duct units. Phenylephrine also potentiates the effects of secretin on cAMP production, and directly increases cytosolic Ca^{2+} and InsP3 in cholangiocytes[27]. Pharmacologic evidence suggests that cholangiocytes express D2 but not D1 or D3 dopamine receptors. Stimulation of these receptors inhibits rather than stimulates secretin-induced ductular secretion and cAMP production. This is thought to be because D2 receptor stimulation co-activates PKC-gamma, whereas gastrin receptor stimulation co-activates PKC-alpha[28]. Thus, although several types of receptors link to Ca^{2+} signaling in cholangiocytes, they can have distinct downstream actions because of differential effects on Ca^{2+} signaling patterns as well as because of differential activation of related signaling pathways.

REGULATION OF SECRETION *VIA* Ca^{2+}

Cholangiocytes comprise less than 5% of normal liver, but play an important role in the process of bile secretion[29,30]. Cholangiocytes can be stimulated to increase bile flow by up to 50%[31], although cholangiocytes typically affect the pH rather than the volume of bile[32]. Two parallel signaling pathways exist to regulate ductular bicarbonate secretion, which are mediated by either cAMP or Ca^{2+}. Evidence in isolated cholangiocytes and bile duct units suggests that cAMP stimulates chloride secretion *via* the cystic fibrosis transmembrane conductance regulator (CFTR), and that bicarbonate secretion then occurs *via* an associated chloride-bicarbonate exchanger. Some evidence in isolated cell systems suggests that Ca^{2+} analogously stimulates chloride secretion *via* a Ca^{2+}-activated chloride channel, and that bicarbonate secretion then occurs *via* an associated chloride-bicarbonate exchanger[33]. Alternative evidence instead suggests that Ca^{2+} stimulates ductular secretion by calcineurin-mediated potentiation of the cAMP pathway[18]. The mechanism of ductular secretion has also been investigated in the intact liver, by bivascular perfusion of the isolated liver *via* both the hepatic artery and the portal vein[32]. This bivascular perfusion model is necessary to use because the blood supply of cholangiocytes is derived *via* the hepatic artery in normal liver. Thus, for example, the effects of secretin on ductular secretion can be readily detected when this hormone is infused *via* the hepatic artery, but are difficult to detect when infused *via* the portal vein[32]. These studies show that ductular bicarbonate secretion induced by the Ca^{2+} agonist ACh depends upon both chloride channels and chloride-bicarbonate exchange. This observation supports the idea that Ca^{2+}-mediated bicarbonate secretion results from serial activation of chloride channels, and then chloride-bicarbonate exchange. However, bivascular perfusion studies show that cAMP-mediated bicarbonate secretion depends upon chloride channels but not chloride-bicarbonate exchange[32]. This observation supports the idea that ductular bicarbonate secretion induced by cAMP may occur directly *via* CFTR, similar to what has been described in intestine[34,35].

REGULATION OF APOPTOSIS *VIA* Ca^{2+}

Apoptosis in all cell types is regulated in part through Ca^{2+} signaling pathways, and there are special considerations in cholangiocytes. Apoptotic pathways are modulated through mitochondrial permeabilization, which in turn is regulated through Ca^{2+} signaling. The interrelationship between mitochondria and Ca^{2+} signaling results from the close proximity between mitochondria and InsP3Rs[36]. As a result of this physical association, mitochondria are subjected to more intense increases in Ca^{2+} than typically occur in cytosol[36]. Excessive increases in mitochondrial Ca^{2+} result in increases in mitochondrial permeability that are associated with leakage of cytochrome c into the cytosol, which leads to apoptosis[37]. Cytochrome c released from mitochondria binds directly to nearby InsP3Rs, which potentiates release of Ca^{2+} from the receptor[38]. This leads to a positive feedback loop that further increases mitochondrial permeability and promotes apoptosis[38]. On the other hand, the anti-apoptotic Bcl-2 family members Bcl-xL and Bcl-2 inhibit apoptosis by inhibiting InsP3-mediated Ca^{2+} release. Bcl-xL decreases expression of InsP3R, which reduces InsP3-mediated Ca^{2+} release in order to protect against apoptosis[39]. Bcl-2 itself increases leakage of Ca^{2+} from the ER, which also has the net effect of decreasing Ca^{2+} signals in both the cytosol and mitochondria[40]. Mcl-1 is the principal Bcl-2 family member in cholangiocytes[41] (Figure 1). Like other Bcl-2 family members, it appears to inhibit apoptosis through effects on Ca^{2+} signaling. Unlike Bcl-xL or Bcl-2, however, Mcl-1 does not affect either InsP3R expression or ER Ca^{2+} stores[42]. Instead, Mcl-1 inhibits Ca^{2+} signaling directly within mitochondria[42].

All three isoforms of the InsP3R can induce apoptosis[11]. However, each isoform has a different propensity to induce apoptosis[11]. Recent evidence suggests that this may be due in part to their differential distribution relative to mitochondria. Specifically, in cells that co-express each InsP3R isoform, the type Ⅲ $InsP_3R$ is most effective in transmitting Ca^{2+} signals to mitochondira as well as in inducing apoptosis[11]. Furthermore, the type Ⅲ isoform co-localizes most strongly with mitochondria at the light level[11]. Thus, the type Ⅲ InsP3R has a particular propensity to form signaling microdomains with mitochondria. The

type Ⅲ $InsP_3R$ is most concentrated in the apical region of cholangiocytes[5], as described above, but little is known about the factors that regulate this subcellular targeting. It also remains unknown whether apical, type Ⅲ InsP3Rs associate with mitochondria in cholangiocytes, or whether such an association is needed to mediate apoptosis in bile ducts.

Ca^{2+} SIGNALING IN HEALTH AND DISEASE

Ca^{2+} signaling is universal among cells, so one might expect altered Ca^{2+} signaling to play an important role in pathogenesis of disease. In fact, the spectrum of human diseases that had been found to result from alterations in Ca^{2+} signaling has been remarkably limited. These have included a mutation in the RyR, which results in malignant hyperthermia[43]; mutations of the Ca^{2+}-ATPase pump, which are associated with deafness[44]; and an animal model of loss of the types Ⅱ and Ⅲ InsP3Rs, which together results in pancreatic insufficiency and failure to thrive[45]. However, it now appears that loss of InsP3Rs is a common event in human cholestatic disorders. Specifically, expression of the type Ⅲ InsP3R is decreased in the cholangiocytes of patients with primary biliary cirrhosis, sclerosing cholangitis, biliary atresia, and ductular obstruction[46]. This loss of InsP3R expression also occurs in animal models of cholestasis, where it is associated with impaired Ca^{2+} signaling, as well as impaired Ca^{2+}-induced secretion in cholangiocytes[46]. However, cAMP-mediated secretion is maintained in cholangiocytes despite the loss of InsP3Rs. Interestingly, the loss of InsP3Rs that is seen in patients with cholangiopathies is not seen in patients with hepatitis C viral infections, even though such infections are associated with inflammation in the region of the portal triad[46]. This suggests that loss of InsP3Rs from cholangiocytes is not merely a nonspecific effect of local inflammation. Together, these findings suggest that Ca^{2+} signaling pathways are necessary for ductular secretion as it occurs *in vivo*, and that loss of InsP3Rs results in cholestasis, although direct support for this hypothesis is lacking.

Just as impaired Ca^{2+} signaling in cholangiocytes is associated with the development of cholestasis, stimulation of Ca^{2+} signals may be beneficial for the treatment of cholestasis. Ursodeoxycholic acid (UDCA) and its taurine conjugate (TUDCA) are of therapeutic benefit in a range of cholestatic disorders[47]. Recent evidence suggests that these but not other bile acids selectively induce hepatocytes to secrete ATP into bile[23]. In addition, luminal perfusion of isolated intrahepatic bile duct segments with ATP increases Ca^{2+} in cholangiocytes and stimulates ductular bicarbonate secretion, while perfusion with TUDCA does not[23]. These findings suggest that UDCA and TUDCA may promote ductular secretion through a paracrine signaling pathway whereby hepatocytes release ATP to activate apical purinergic receptors on cholangiocytes, which link to Ca^{2+}-mediated secretion of chloride and bicarbonate into bile. Further work is needed to understand how these bile acids induce hepatocytes to secrete ATP, and to demonstrate that this action is in part responsible for their therapeutic effects.

CONCLUSION

Ca^{2+} signaling is a ubiquitous mechanism for regulation of cell function. A range of neuroendocrine and paracrine messenger molecules exert their effects on cholangiocytes through Ca^{2+} signaling. It is already established that these effects include regulation of bicarbonate secretion and regulation of apoptosis. By extension from what has been shown in other cell types, it is likely that Ca^{2+} signaling also regulates other aspects of ductular secretion, including fluid secretion and vesicular targeting and exocytosis, as well as other aspects of cell proliferation, including gene transcription and progression through the cell cycle. Future areas of investigation are likely to more fully identify the range of actions of Ca^{2+} in cholangiocytes. Such information may in turn reveal specific ways in which impaired Ca^{2+} signaling leads to cholangiopathies and related disease states, which then may lead to targeted therapies that are based on correcting such impairments.

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TOPIC HIGHLIGHT

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Nervous and Neuroendocrine regulation of the pathophysiology of cholestasis and of biliary carcinogenesis

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Abstract

Cholangiocytes, the epithelial cells lining the biliary ducts, are the target cells in several liver diseases. Cholangiopathies and cholangiocarcinoma generate interest in many scientists since the genesis. The developing mechanisms, and the therapeutic tools of these diseases are still undefined. Several studies demonstrate that many hormones, neuropeptides and neurotransmitters regulate malignant and non-malignant cholangiocyte pathophysiology in the course of chronic biliary diseases. The aim of this review is to present the findings of several studies published in the recent years that contributed to clarifying the role of nervous and neuroendocrine regulation of the pathophysiologic events associated with cholestasis and cholangiocarcinoma development. This manuscript is organized into two parts. The first part offers an overview of the innervation of the liver and the origin of neuroendocrine hormones, neurotransmitters and neuropeptides affecting cholangiocyte function and metabolism. The first section also reviews the effects played by several neuroendocrine hormones and nervous system on cholangiocyte growth, survival and functional activity in the course of cholestasis. In the second section, we summarize the results of some studies describing the role of nervous system and neuroendocrine hormones in the regulation of malignant cholangiocyte growth.

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Key words: Cholangiocyte; Neuroendocrine hormones; Neurotransmitters; Neuropeptides; Cholestasis; Nervous System; Biliary carcinogenesis; Pathophysiology; Cholangiocarcinoma; Proliferation

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INTRODUCTION

Cholangiocytes are the epithelial cells that line the intrahepatic biliary tree. Their physiologic role is to modify the bile of canalicular origin, through a wide array of absorbtive and secretive processes[1,2]. Indeed, although in normal conditions they represent approximately the 4%-5% of the liver mass, they contribute for at least the 10%-30% of the bile flow[1].

Cholangiocytes are the target of chronic diseases, termed cholangiopathies[3,4], that are commonly characterized by dysregulation of the balance between cell growth and survival. In the course of such diseases there is impaired proliferative response to duct injury and increased cell death by apoptosis, that leads to vanishing of bile ducts[3] and functional liver failure at the end stage. Cholangiopathies thus represent a daily challenge for the clinicians, since definitive medical treatments are not available yet. As a consequence, 20% of liver transplants among adults and 50% of those among pediatric patients are due to these disorders[5].

The malignant transformation of cholangiocytes gives origin to cholangiocarcinoma[6]. Cholangiocarcinoma is often diagnosed at late stages; its surgical resection has given very limited success, as response of this neoplasm to conventional chemotherapy[6] is minimal. These features make cholangiocarcinoma a malignancy characterized by a very poor prognosis. In addition, the incidence and prevalence of cholangiocarcinoma are increasing worldwide[7].

Many of the problems that arise in the management

of cholangiopathies and of cholangiocarcinoma can at least in part be ascribed to the poor knowledge about their pathophysiology. Very little is in fact known on which factors are able to affect cholangiocyte biology, with particular regard to those endogenous molecules or systems that affect either cholangiocyte proliferation, survival or functional activity[4].

Bile acids are endogenous factors that have been historically investigated more in this regard, and their properties in modulating cholangiocyte biology and cholangiocarcinoma development are now unanimously accepted[8-13]. More recently, several studies demonstrated that many hormones, neuropeptides and neurotransmitters are able to affect malignant and non malignant cholangiocyte biology as well. This data showed that the biliary epithelium is similar to the gastric or intestinal epithelia, for which the key role played by nerves and neuroendocrine hormones has been known for years[14]. Moreover, it has been shown that in the course of chronic cholestasis, cholangiocytes acquire a neuroendocrine phenotype, that is not proper for these cells in normal conditions[15]. Immunohistochemical studies have also reported that many biliary tract carcinomas (up to the 70% of the cases) show a neuroendocrine differentiation[16,17].

The purpose of this review is therefore to summarize the evidences from the several studies published in the recent years that contributed to clarify the role of nervous and neuroendocrine regulation of the pathophysiologic events associated with cholestasis and cholangiocarcinoma development. In particular, we will focus on how these factors affect cholangiocyte cell biology. We suggest those who might be interested to learn the effects of nerves and neuroendocrine hormones on other liver cell type biology to other reviews recently published on this subject[18-20].

ORIGIN OF NEUROENDOCRINE HORMONES, NEUROTRANSMITTERS AND NEUROPEPTIDES AFFECTING CHOLANGIOCYTES

Neuroendocrine hormones are secreted by neuroendocrine cells, that are diffusely present in the whole gastrointestinal tract, in particular the stomach, small bowel[21], as well as pancreas[22]. If these cells are considered the main source of the neuroendocrine hormones, in this review some studies suggest that cholangiocytes them-selves can synthesize some of these peptides in the course of cholestasis.

The liver is innervated by both sympathetic and parasympathetic nerves, whose fibers are located around the hepatic artery, portal vein, intrahepatic and extrahepatic bile ducts[23,24]. Sympathetic nerves originate from the celiac ganglion, whereas the parasympathetic from the vagus nerve[23,24]. Besides catecholamines and acetylcholine, autonomic fibers that innervate the liver can also release other neurotransmitters, like neuropeptide Y (NPY)[25,26], calcitonin gene related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide (VIP), enkephalin and bombesin[27-30]. Nerve terminations mostly follow the vascular structures of the portal tract, and have been identified around bile ducts and vessels. Many of the above mentioned neurotransmitters have been shown to modulate intrahepatic hemodynamics[31-33].

Table 1 Neuroendocrine hormones affecting cholangiocyte biology

Hormone	Receptor	Effect on cholangiocyte biology	Reference
Secretin	SR	Stimulates HCO_3^- secretion through the increase of cAMP/PKA and opening of CFTR	1, 34-43
Somatostatin	$SSTR_2$	Counteracts the effect of secretin; stimulates bile absorption	44, 45
Insulin	IR	Counteracts the effect of secretin	46
ET-1	ET_A-ET_B	Counteracts the effect of secretin; reduces the SR expression	47
VIP	unspecified	Increases HCO_3^- and water secretion	48, 49
Bombesin	unspecified	Increases HCO_3^- and water secretion	50
Gastrin	CCK-B/ gastrin	Counteracts the effect of secretin; reduces cell proliferation	40, 51
Estrogens	ERα-ERβ	Stimulate cell proliferation	52-55
Serotonin	$5HT_{1A}$-$5HT_{1B}$	Synthesized and secreted by cholangiocytes in the course of cholestasis. Counteracts the effect of secretin and reduces cell growth	58
GH/IGF-1	GH-R-IGF-1R	Stimulate cell proliferation	59

NERVOUS AND NEUROENDOCRINE REGULATION OF THE PATHOPHYSIOLOGY OF CHOLESTASIS

Regulation of cholangiocyte growth, survival and functional activity by neuroendocrine hormones (Table 1)

The close link between cholangiocytes and neuroendocrine hormones has its basis on the early studies that showed the hormone secretin as the most potent regulator of cholangiocyte functional activity[34,35]. In 1988, Alpini *et al* demonstrated that in a rat model of cholestasis (induced by bile duct ligation, BDL) the infusion of secretin was associated with a marked increase of the bile flow and bicarbonate biliary excretion[35]. After that "landmark" manuscript, a large series of investigations later defined the intracellular mechanisms by which secretin induces such a potent functional stimulus. It is now known that secretin stimulates ductal secretion[1,34-36] by selective interaction with secretin receptors, expressed only by cholangiocytes in rat liver[37]. The interaction of secretin with its own receptors[37] leads to an increase in intracellular cAMP levels[1,36,38-40], activation of PKA[41], opening of CFTR Cl^- channels[42] with activation of the Cl^-/HCO_3^- exchanger[1,36,41,43], which leads to secretion of bicarbonate into bile[35].

Other neuroendocrine hormones were then discovered to affect cholangiocyte choleretic activity. One of the first molecules studied was somatostatin. It was demonstrated that cholangiocytes express the $SSTR_2$ receptor; the interaction of somatostatin with this receptor markedly diminished the effect of secretin on the biliary excretion

of water and bicarbonate by cholangiocytes in cholestatic conditions[44]. Such an effect of somatostatin was due to the fact that the activation of the $SSTR_2$ receptor prevented the increase of the adenylyl-cyclase elicited by secretin[44]. In later studies conduced in IBDUs isolated from wild type and $SSTR_2$-knock out mice, it was also found that somatostatin not only reduces cholangiocyte choleresis, but it also stimulates ductal bile absorption[45].

Cholangiocytes also express at the apical pole the receptor for insulin[46]. Upon its activation, a marked reduction of the secretin-induced choleresis was observed, both if the hormone was administered *in vivo* to BDL rats and if microinjected into the lumen of IBDUs isolated from animals with cholestasis. It was observed that the activation of the insulin receptor determined a cascade of intracellular events that resulted in the inhibition of secretin-stimulated cAMP and PKA activity. Such a chain of events seemed to have its core event in the enhancement of the intracellular Ca^{2+} levels and the consequent activation of the Ca^{2+}-dependent PKCα[46]. An effect similar to the one of insulin has been described for endothelin-1 (ET-1), which interacts with the specific receptors expressed in the biliary epithelium (ET_A and ET_B) and blunts the secretin-induced choleresis of the BDL rat and reduces the expression of the secretin receptor on cholangiocytes[47].

In contrast to somatostatin and insulin, vasoactive intestinal polypeptide (VIP) and bombesin have been found to be able to enhance cholangiocyte choleresis. Both the hormones induced a potent fluid and bicarbonate excretion in IBDUs, but not in hepatocytes, isolated from normal and cholestatic rats. Interestingly, neither VIP nor bombesin had any significant effect on modulating intracellular cAMP levels[48-50]. Detailed pH studies indicated that the underlying intracellular mechanism, at least for bombesin, is its ability to stimulate the activity of Cl^-/HCO_3^- exchange in association with a counterbalancing secondary activation of electrogenic Na^+/HCO_3^- symport[50].

In more recent studies, increasing evidence regarding the ability of neuroendocrine hormones to affect cholangiocyte growth, and functional activity, hvee been reported.

If the infusion of gastrin to BDL rats is associated with the reduction of the choleretic response to secretin by cholangiocytes[40], its chronic administration through an intraperitoneal minipump resulted not only in reduced functional activity, but also in a marked decrease of the bile duct mass[51]. Cholangiocytes indeed express the CCK-B/gastrin receptors[51], which, upon activation, elicit the intracellular Ca^{2+} release, the increase of IP_3 levels, and the membrane translocation (e.g. activation) of the Ca^{2+}-dependent PKCα[51]. In turn, the gastrin-activated PKCα, as above mentioned, is able to interfere with the secretin signaling and modulate the adenyl-cyclase activity, thus reducing the intracellular cAMP levels and PKA activity. These observations, demonstrated that bile acids also activate this intracellular pathway to modulate cholangiocyte growth[8], contribute to elucidate which intracellular signalings sustain the proliferative response of cholangiocytes to cholestasis and by which mechanisms it is possible to modulate them. Moreover, since cAMP/PKA resulted the key molecules implicated in cholangiocyte proliferation, this helped to explain the association between increase of duct mass and enhanced biliary choleresis[8,35].

A major contribution to this field of research has been given by the studies that clarified the effects of estrogens on the biliary epithelium. Both the estrogen receptor (ER) α and β were observed in cholangiocytes[52], their expression being up-regulated after BDL[53]. When cholangiocytes were stimulated *in vitro* with 17-β-estradiol, their proliferation was markedly increased, as a consequence of the ER-dependent activation of Src/Shc/ERK1/2 intracellular pathway[54]. To demonstrate the physiological and pathophysiological relevance of estrogens on cholangiocyte proliferative response to cholestasis, when BDL male rats were treated *in vivo* with antiestrogens like tamoxifen or Ici 182 780[52] or when BDL female rats were subjected to ovariectomy[53], the growth of the biliary tree was blunted and the biliary epithelium underwent programmed cell death by apoptosis[52,53]. This evidence suggested that estrogens are required for a separative response of the biliary tree to injury. These studies produced in the last few years by Alvaro represent a significant change in the knowledge of the role played by the neuroendocrine system on the biliary cell biology. Indeed, primary biliary cirrhosis (PBC), the most common from of cholangiopathies[4], is much more frequent in women than in men, and has its clinical outcome typically after menopause, when the endogenous estrogen levels suddenly drop[55]. Therefore, these observations by Alvaro seem to provide the biological confirmation of the role of estrogens in the progression of PBC, a role that was, earlier, only hypothesized on the basis of epidemiological data. To further support this concept, Alvaro also demonstrated that the ER expression in cholangiocytes is markedly reduced in late stages of PBC[55]. Altogether, Alvaro's studies made clear that gaining knowledge on the role of neuroendocrine hormones on cholangiocyte biology might also be an effective strategy to further understand the pathophysiology of the cholangiopathies and thus eventually to design novel therapeutic strategies.

The neuroendocrine hormone serotonin has been hypothesized to be involved in the genesis of certain clinical features of PBC, like fatigue and pruritus[56,57]. Interestingly, it has been recently shown that cholangiocytes express the serotonin 1A and 1B receptors[58]. When they are activated by selective agonists, the proliferation of BDL cholangiocytes are dramatically reduced, both *in vivo* and *in vitro*[58], in association with the loss of the response to secretin of the markers of cholangiocyte functional activity[58], like the bile flow, the bicarbonate excretion and the intracellular cAMP levels[34]. Such an effect seems more to be mediated by the cross-talk between the Ca^{2+} and the cAMP/PKA signalings. If the serotonin 1A and 1B receptors are activated, there is an increase of Ca^{2+}, IP_3 and PKCα levels, with the consequent reduction of the intracellular cAMP levels and PKA activity. Most interestingly, it was observed that hyperplastic cholangiocytes isolated from BDL rats are able to synthesize and secrete serotonin.[58] If, *in vitro* or *in vivo*, cholangiocyte-secreted serotonin is neutralized,

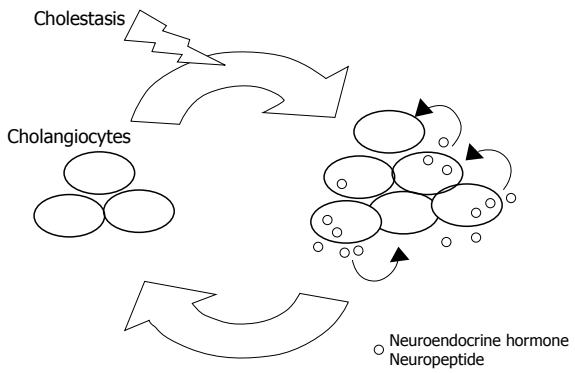


Figure 1 Proposed working model for the existence of an autocrine/paracrine loop of neuroendocrine hormones and neuropeptides that regulate the proliferative response of cholangiocytes to cholestasis. A chronic cholestatic condition induces cholangiocyte proliferation and their transdifferentiation in neuroendocrine-like cell. This allows the biliary epithelium to start to synthesize and secrete a number of peptides that aim to counterbalance the effects of cholestasis itself on cell growth, as a sort of negative feed-back.

Table 2 Neurotransmitters/neurotrophins affecting cholangiocyte biology

Peptide	Receptor	Effect on cholangiocyte biology	Reference
Acetylcholine	M3	Potentiates the effect of secretin. Required for cholangiocyte response to BDL: sustains cholangiocyte proliferation and prevents apoptosis	43, 60, 61, 64, 65
Epinephrine/ Norephinephrine	α_1	Potentiates the effect of secretin	67
Epinephrine/ Norephinephrine	β_1-β_2	Required for cholangiocyte response to BDL: sustains cholangiocyte proliferation and prevents apoptosis	70
Dopamine	D2	Inhibits secretin-induced ductal secretion	73
NGF	TrkA	Secreted by hyperplastic, cholestatic cholangiocytes; sustains the rpoliferative response to BDL	74

cholangiocyte proliferation in response to cholestasis further increases[58]. This study, therefore, proposes an additional novel working model (as shown in Figure 1): in the course of cholestasis, cholangiocytes acquire a neuroendocrine phenotype that allow them to synthesize some neuroendocrine hormones, like serotonin. Once secreted, these hormones aim to counterbalance the excessive proliferative response of the biliary epithelium. It is thus possible to postulate the existence of an autocrine/paracrine loop of peptides and neuroendocrine hormones that modulate with a negative (or an eventually positive) feed-back the response of cholangiocytes to liver injury[58]. The investigation of the presence of other peptides and hormones that are likely to participate with serotonin in this autocrine/paracrine loop might thus be needed to understand certain still obscure clinical aspects of cholangiopathies.

Recent evidence demonstrated that a "stimulatory" autocrine/paracrine loop should also exist. It has been found that cholangiocytes are the target of the growth hormone (GH)-insulin like growth factor (IGF)-1 axis[59]: GH induces IGF-1 expression and release in isolated cholangiocytes, with the consequent stimulation of cell growth by IGF-1[59].

Regulation of cholangiocyte growth, survival and functional activity by neurotransmitters (Table 2)

A number of studies showed that cholinergic nerves regulate bile secretion[60,61]. In bile-fistula dogs with interrupted enterohepatic circulation, distal stimulation of the vagus nerve increases bile bicarbonate secretion, whereas vagotomy decreases basal bile flow and bicarbonate output[60,61].

In early studies[62] it seemed that cholangiocytes might express both the M1 and the M3 acetylcholine (ACh) receptor subtypes and that the cholinergic agonist, carbachol, stimulates bile flow of the isolated perfused rat liver. These observations were not confirmed by successive investigations. In 1996, Nathanson *et al* demonstrated that, in accordance with the concept that cholangiocytes, but not hepatocytes, express ACh receptors, ACh did not affect the functions of hepatocytes, but elicited Ca^{2+} increase and oscillation in IBDUs, due to both an influx of extracellular Ca^{2+} and the mobilization of thapsigargin-sensitive Ca^{2+} stores[63]. A year later, Alvaro *et al* showed that cholangiocytes express, at the basolateral domain, M3 (but not M1 and M2) Ach receptors.[43] In that study it was shown that ACh has no effect on the basal activity of the Cl^-/HCO_3^- exchanger, but it significantly potentiates the stimulatory effect of secretin on this anion exchanger[43]. Presumably, the significance of ACh-regulation of bicarbonate secretion by bile ducts is to be researched in the need of bicarbonate-enriched secretion in the small intestine during the digestive phase, when, indeed, the parasympathetic system activity is high. By selectively interacting with M3 receptor subtypes, ACh induces a Ca^{2+}-calcineurin mediated potentiation of the secretin-induced adenylyl cyclase activity, which leads to activation of the Cl^-/HCO_3^- exchanger with bicarbonate secretion into bile[43]. Furthermore, recent studies have shown that ACh sustains cholangiocyte proliferation, since interruption of the cholinergic innervation by vagotomy induces a marked decrease in total bile duct mass caused by both impaired cholangiocyte proliferative capacity and intracellular cAMP levels, and enhanced cell death by apoptosis[64,65]. These studies also show that maintenance of intracellular cAMP levels (by chronic administration of forskolin) prevents the effects of vagotomy on cholangiocyte apoptosis, proliferation and secretion[65].

There is growing information regarding the role of adrenergic and dopaminergic innervation in the regulation of cholangiocyte proliferation and secretion. An intact sympathetic innervation is required for hepatocyte and cholangiocyte proliferation following partial hepatectomy[66]. Recent studies demonstrated that: (1) cholangiocytes from BDL rats express alpha-1, alpha-2, beta-1 and beta-2 adrenergic receptors; and (2) alpha-1 (but not beta-1) adrenergic receptor agonists increase secretin-stimulated ductal secretion[67]. Similar to what is shown in the gut, adrenergic innervation may play a role in counterbalancing the stimulatory effects of cholinergic

nerves[65] on ductal bile secretion in chronic cholestatic liver diseases.

In support of the concept that adrenergic innervation plays an important role in the regulation of cholangiocyte functions, administration of a single intraportal injection of 6-hydroxidopamine, which induces degeneration of dopaminergic terminal fibers[68,69], blunts the cholangiocyte functional and proliferative response to cholestasis and induces cell death by apoptosis[70]. Chronic administration of clenbuterol (a beta-2 adrenergic agonist)[71] and dobutamine (a beta-1 adrenergic agonist)[72] prevents the decrease in cAMP levels and secretion induced by 6-OHDA, maintains cholangiocyte proliferation and decreases cholangiocyte apoptosis due to 6-OHDA[70]. Furthermore, it has been shown that cholangiocytes express the D2 (but not the D1 and D3) dopaminergic receptors and that the D2 dopaminergic agonist, quinelorane inhibits secretin-induced ductal secretion in BDL rats through activation of the Ca^{2+}-dependent PKC gamma (but not PKC alpha, beta I and II) and inhibition of secretin-stimulated cAMP levels and PKA activity[73].

The relationship between the biliary epithelium and nerves appears to be more than what was thought at the beginning. Cholangiocytes express the receptor for neurotrophin Nerve Growth Factor (NGF), the activation of which strongly promotes biliary cell growth[74]. Moreover, it has been found that cholangiocytes themselves can produce and secrete NGF and that when this neurotrophin is immunoneutralized the growth of the biliary tree in the BDL rat is strongly diminished[74].

Altogether, these data suggest that the autonomic innervation plays a substantial role in the regulation of cholangiocyte biology, thus enlightening the need of studying whether the liver denervation after transplantation might affect the functions of the grafted biliary tree. Of major interest would also be to investigate whether other endogenous factors, instead of nerves, can support cholangiocyte functions in the denervated organ. In this view, some evidence suggest that bile acids could be important. It has been found that administration of taurocholic[64] or ursodeoxycholic acid[75] counteracts the loss of bile ducts induced by cholinergic denervation in the BDL rat. Similarly, taurocholic acid administration also prevents the loss of bile ducts induced by adrenergic denervation[76].

NERVOUS AND NEUROENDOCRINE REGULATION OF BILIARY CARCINOGENESIS

Cholangiocarcinoma, the primary cancer that originates from the epithelial cells lining the bile ducts, is a devastating malignancy characterized by poor prognosis and high mortality[6,77]. The only curative treatment, even if only possible at an early stage of this neoplasm, is surgical. However, patients often present this tumor at an advanced stage, when a curative surgery is unlikely. In fact, at the time of diagnosis, more than two-thirds of patients affected by biliary tract cancer present as an unresectable disease[78] and patients with an operable tumor have a high rate of recurrence. Overall survival rate, including resected patients is quite poor, with less than 5% of patients surviving 5 years, a rate which has remained unchanged over the past 30 years[77]. Chemotherapy and radiation therapy have been used in an attempt to control this disease and improve survival and quality of life of patients with unresectable, recurrent and metastatic cholangiocarcinoma, but these therapies have not shown to be effective in prolonging long-term survival. For these reasons there is a compelling need to discover novel molecules and agents to target the cholangiocarcinoma cells and to regulate their destructive growth[79]. There is growing information regarding the role of nerves and neuropeptides as modulators of cholangiocyte function and metabolism[14]. Moreover, several reports suggest that nervous stimuli are involved in the regulation of growth of biliary malignancies[16,17,80].

Table 3 Neuroendocrine hormones affecting cholangiocarcinoma cell biology

Hormone	Receptor	Effect on cholangiocarcinoma cell biology	Reference
Estrogens	ER	Stimulated SK-ChA-1 human cholangiocarcinoma cell growth	81
		Tamoxifen induced dose-dependent growth inhibition of OZ and SK-ChA-1 human cells *in vitro*; reduced growth of a SK-ChA-1 tumor cell xenografts implanted in athymic nude mice	81
		Tamoxifen stimulated SK-ChA-1 human cholangiocarcinoma apoptotic cell death	82
		Tamoxifen induced human cholangiocarcinoma cell apoptosis *in vitro* and inhibited tumor xenograft growth after pretreatment with IFN-gamma	83, 84
Gastrin	CCK-B /gastrin	Inhibited Mz-ChA-1, HuH-28, and TFK-1 human cell lines proliferation and induced Mz-ChA-1 cell apoptosis	90
CCK	CCK	Reduced the growth of SLU-132 human tumor xenografts implanted in nude mice; stimulated the release of carcinoembryonic antigen (CEA) by SLU-132 cells	93

Regulation of malignant cholangiocyte growth by neuroendocrine hormones (Table 3,Table 4)

The involvement of neuroendocrine system in regulating cholangiocarcinoma cell proliferation has been suggested by several studies. Tamoxifen, an estrogen antagonist, cause a dose-dependent decrease of proliferation of two human cholangiocarcinoma cell lines *in vitro*[81]. On the other hand, 17-β estradiol stimulate human cholangiocarcinoma cell growth *in vitro*[81]. In addition, tamoxifen induced a growth reduction of a cholangiocarcinoma tumor implanted in athymic nude mice[81]. Tamoxifen exerts its inhibitory effect in human cholangiocarcinoma cells by stimulating apoptotic cell death and this is likely mediated through the Fas/APO-1 (CD95) signaling pathway via a calmodulin-dependent mechanism[82].

A further study showed that tamoxifen exposure to human cholangiocarcinoma after pre-treatment with

IFN-gamma allows for induction of apoptosis *in vitro* and significant inhibition of tumor growth[83]. Thus, the combination of IFN-gamma and estrogen antagonists, including tamoxifen, could be useful to have a sustained and valid inhibitory effect on cholangiocarcinoma cell growth[83,84].

Gastrointestinal polypeptide hormones regulate the growth of various normal gastrointestinal tissues as well as certain visceral cancers. Gastrin, which belongs to the superfamily of cholecystokinin receptors (CCK-A, CCK-B/gastrin receptors), is a trophic factor within the normal gastrointestinal tract and is also a mitogen for a number of gastrointestinal and non-gastrointestinal tumors such as gastric, colonic and pancreatic[85-88]. In contrast, CCK-B/gastrin receptor signaling in the human pancreatic cell lines MiaPaca-2 and Panc-1 leads to inhibition of cell growth[89]. It has been shown that malignant cholangiocytes express gastrin receptors[90] and that gastrin, interacting with CCK-B/gastrin receptors, inhibited the proliferation of Mz-ChA-1, HuH-28 and TFK-1 cholangiocarcinoma cell lines, also inducing apoptosis in Mz-ChA-1 cells by activation of the Ca^{2+}-dependent PKC-α signaling[90]. The blockage of CCK-B receptors did not totally reverse the inhibitory effect of gastrin on Mz-ChA-1 cell growth. This finding could be explained by the fact the gastrin may exert its effect not only through CCK-B/gastrin receptors, but also through other receptor types, as suggested by studies in other carcinomas cell lines (*e.g.* colonic cancer cells)[91]. This partial inhibitory effect by CCK-B/gastrin receptor inhibitors might also be due to altered processing of the CCK/B receptor by malignant transformed cells, as previously described[92]. A previous study by Hudd *et al* showed that human cholangiocarcinoma cells expressed CCK receptors and chronic treatment with CCK octapeptide reduced the growth of human cholangiocarcinoma tumors implanted in nude mice[93].

Somatostatin receptors (SS), most commonly the SS receptor type 2 (SSTR2), have been described in several neuroendocrine and epithelial malignancies[94], such as in malignant cholangiocytes[95,96]. Studies demonstrated that somatostatin and its analogues inhibit *in vitro* cholangiocarcinoma cell proliferation. Indeed, chronic administration of lanreotide, a long-acting SS analogue, reduces the growth of human cholangiocarcinoma cells when implanted in athymic mice[95,96] and the inhibitory effect of somatostatin in cholangiocarcinoma cell growth was accompanied by no changes in cellular cyclic adenosine monophosphate (cAMP) or calcium intracellular levels. Furthermore, Zhao *et al* showed that octreotide inhibits cholangiocarcinoma cells growth through G0/G1 cell cycle arrest rather than through the process of apoptosis. These effects were partially mediated by enhancing the expression of $p27^{kip1}$, and by decreasing the amounts of cyclin E-CDK2 complex[95]. Taken together, these findings suggested possible use of SS analogues in the diagnosis or therapy of cholangiocarcinoma. However, a subsequent phase II study showed absence of therapeutic efficacy of the somatostatin analogue lanreotide in the treatment of advanced primary hepatic cholangiocellular cancer and gallbladder adenocarcinoma, despite *in vivo* somatostatin-receptor expression[97].

Table 4 Neuroendocrine hormones affecting cholangiocarcinoma cell biology

Hormone	Receptor	Effect on cholangiocarcinoma cell biology	Reference
Somatostatin, analogues (octreotide, lanreotide)	$SSTR_2$	Inhibited human cholangiocarcinoma cell proliferation *in vitro* and reduced human cholangiocarcinoma cell growth implanted in athymic mice	96
		Inhibited RBE, NEC, QBC939, and SSP-25 cell proliferation *in vitro* through cell cycle arrest and QBC939 xenografts growth	95
GABA	$GABA_{A,-B,-C}$	Inhibited human Mz-ChA-1, HuH-28 and TFK-1 cell proliferation *in vitro*; reduced malignant cholangiocyte migration. Reduced Mz-ChA-1 xenograft tumor growth implanted in athymic mice	98
NPY	NPY-Y5	Inhibited Mz-ChA-1 cell proliferation *in vitro*	99

Liver represents the most important site of GABA synthesis and metabolism outside the central nervous system. Recently, our group demonstrated that cholangiocarcinoma cells express $GABA_{A,-B,-C}$ recep-tors and respond to GABA stimulation with growth inhibition. GABA inhibitory effect on malignant cholangiocyte proliferation was evident *in vitro* and also *in vivo*, by reducing the growth of cholangiocarcinoma tumors injected subcutaneously in nude mice. Moreover, GABA has been shown to be able to inhibit malignant cholangiocyte migration, a peculiar characteristic of the cholangiocarcinoma cells[98].

Another neurotransmitter available in the liver parenchyma and biliary tract is the neuropeptide Y (NPY)[99]. Recent preliminary data from our group showed that NPY inhibits cholangiocarcinoma growth by interaction with a G-protein coupled receptor by Ca^{2+} dependent modulation of Src/ERK1/2 phosphorylation[99].

Regulation of malignant cholangiocyte growth by neurotransmitters (Table 5)

Autonomic nervous system regulates the growth of several tumors[100-102]. For example, alpha-blockers, terazosin and doxazosin, suppress prostate growth by inducing apoptosis among the epithelial cells in the benign and malignant prostate[103]. Also, phenylephrine reduced $HepG_2$ cell growth through $alpha_{1B}$-adrenergic receptors activation[104]. Moreover, activation of a β-2 adrenergic receptor/Gsα fusion protein leads to the inhibition of cAMP-sensitive S49 lymphoma and carcinoma carB cells proliferation *in vitro*[105].

A specific role of sympathetic nervous system in the regulation of cholangiocarcinoma growth has been described by Kanno *et al*, that showed that cholangiocarcinoma cell lines Mz-ChA-1 and TFK-1 express the α_{2A}-, α_{2B}-, α_{2C}- adrenergic receptor subtypes[80]. Furthermore, stimulation of malignant cholangiocytes by UK14, 304, an α_2-adrenoreceptor agonist, causes up-regulation of cAMP, which inhibits EGF-induced MAPK activity through

Table 5 Neurotransmitters/neurotrophins affecting cholangiocarcinoma cell biology

Peptide	Receptor	Effect on cholangiocarcinoma cell biology	Reference
Epinephrine/ Norephinephrine	α_2	UK 14,304 inhibited human Mz-ChA-1 and TFK-1 cell proliferation *in vitro*	[80]
Acetylcholine	M1	Carbachol produced an increase of IP3 and Ca^{2+} intracellular levels in Mz-ChA-1 cells	[62]

an acute increase of Raf-1 and sustained activation of B-Raf[80], thus reducing tumor cell proliferation. Since inhibition of cholangiocarcinoma cell growth through activation of α_2-adrenergic receptor occurred downstream to Ras, this study suggested that adrenergic stimulation or other stimulants of cAMP may overcome the Ras mutations present in malignant cholangiocytes and offer a new therapeutic approach in patients with cholangiocarcinoma[80].

Other studies showed that Mz-ChA-1 cells express muscarinic acetylcholine (ACh) receptors[62]. In support of the hypothesis that such receptors modulate cholangiocarcinoma cell growth, stimulation of malignant biliary cells with carbachol, a muscarinic acetylcholine receptor agonist, triggers IP_3 formation and a subsequent increase of intracellular Ca^{2+} levels[62]. Even if several studies show IP_3 and Ca^{2+} levels play an important role in the inhibition of cholangiocarcinoma cell growth after stimulation with several molecules[13,90,98]. the specific action of parasympathetic nervous system in modulating cholangiocarcinoma cell growth is still unknown.

However, recent data showed that muscarinic receptors are directly activated by other molecules. In fact, recent studies showed that bile acids, interacting with M3 Ach receptors, transactivate EGFR and stimulate colon cancer cell proliferation by inducing p90RSK phosphorylation via a calcium-, MEK- and MAPK-dependent pathways[106].

Recent findings suggest that cholangiocarcinoma and other liver tumors could arise from activated hepatic progenitor cells[107-109]. Several evidences indicate that hepatic progenitor cell activation in diseased liver is regulated by neural and neuroendocrine factors such as vagal innervation[110,111], suggesting an important involvement of autonomic nervous system in developing biliary carcinogenesis.

SUMMARY AND FUTURE PERSPECTIVES

The amount of evidences indicating the substantial role of neuroendeocrine hormones, neuropeptides and neurotransmitters in modulating malignant and non malignant cholangiocyte biology is rapidly increasing. There is still much to understand on how actually these peptides create a network that affects the response of biliary cells to liver injury. On the other hand the other frontier is no doubt represented by the identification of the most proper way of modulating such a network of peptides. Will we be able to interfere with it in order to divert or relent the progression of cholangiopathies and cholangiocarcinoma?

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Bioinformatic approach for understanding the heterogeneity of cholangiocytes

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Abstract

It is remarkable that microarray technologies have nearly reached a pinnacle. Establishment of further analysis and management of enormous data derived from microarray technology is currently the highest priority. The heterogeneous functions of cholangiocytes regulate the pathophysiology of the biliary epithelium in relation to secretory, proliferative and apoptotic activities. Distinct expression profiles of two murine cholangiocyte lines, termed small and large have been revealed by microarray analysis. The features of the two cholangiocyte cell lines, categorized partly according to gene ontology, indicate the specific physiological role of each cell line. The large cholangiocytes are characterized as "transport" and "immune/ inflammatory responses". In contrast, small cholangiocytes are associated with properties of limited physiological functional ability and proliferating/migrating potential with specific molecules like Eph receptors, comparable to mesenchymal cells. 'Omic study will be of great help in understanding the heterogeneity of cholangiocytes.

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Key words: Cholangiocytes; Heterogeneity

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INTRODUCTION

Several research avenues have established novel scientific theories. We initially examined phenotypic manifestations of various forms of induction, analogy and abduction. Subsequent preliminary studies resulted in a firm-working hypothesis. We then tested this hypothesis in order to establish our theoretical groundwork. However, recent advances in the field of bioinformatics have become routine, especially in regards to the initial speculative step in designing a rational approach. Since the initial DNA microarray analysis experiment[1], microarray has developed rapidly and is now well established, with manufacturers meeting market demand[2]. The number of scientific papers with "microarray" as a key word steadily increased on PubMed from 2001 to 2004, reaching a total of 8603 papers. Moreover, an enormous quantity of raw data potentially contained in "microarray" papers is anticipated in the near future, and the estimate is based on the number of the genes analyzed in each paper (e.g. 30 000-40 000 genes expressed in mammalian cells). Therefore, it is essential for future advances to reliably preserve a database containing all the raw data. The establishment of data analysis system as well as data mining and data sharing, is anticipated to be the highest priority in this field. We will focus on such issues pertaining to general microarray experiments as well as the methods and the results of microarray analyses designed to characterize cholangiocytes in this chapter.

MICROARRAY ANALYSIS

We will simply give an overview of the current status, analytic method and data-sharing aspects of microarray analysis, partly in accordance with the description of Knudsen[3]. One of the currently popular microarray systems is the Affymetrix DNA Chip®, which employs *in situ* oligonucleotide synthetic technology as well as mass-production compatible to that of silicon chips. Several microarrays and expression analysis services are commercially available from several companies including Amersham, Clontech (TAKARA), Invitrogen and so on. As in other experiments, the quantity of RNA samples should be standardized, otherwise correction steps will be necessary for the evaluation of the results by

"scaling" generally employing internal controls. Moreover, verification of the linearity of spot intensity-sample quantity curves for all applied spots is also necessary in microarray experiments. The companies supplying ready-made products have overcome these problems and established quality control, making their products popular with researchers applying commercial products to limited numbers of samples. Although array technologies at the bench have nearly reached a zenith during the past several years, there are some in silico systems under development. The parametric *t*-test and non-parametric Wilcoxon's rank-sum/ Mann-Whitney tests are the most popular methods applicable to test the significance of differences between pairs of samples. The normality of each spot intensity for the samples becomes an issue if the *t*-test or ANOVA is used with a routine microarray having thousands of spots. The most conservative statistical method, Bonferroni's correction, which defines the level of significance more strictly according to the number of tested items, might be applicable even to microarray experiments[4]. Even if we employ other less conservative approaches[5], we may need to verify the differential expressions of the specific molecules by other methods in some cases. Several software programs are available, including some that are free like Cluster (http://rana.lbl.gov/EisenSoftware.htm)[6], for analysis of microarray data. Cluster analysis categorizes each gene into distinct clusters according to Euclidian distance based on the variation among the samples at multiple time-points or under the different conditions. Each gene in a cluster shows a similar expression pattern under different conditions, which may indicate a common transcription pathway, RNA degradation process, and/ or similarities of gene function in a cluster. In other words, microarray technology holds promise for elucidating the complex regulatory mechanisms of RNA transcription as well as unknown gene functions. Every gene is currently being systematically defined, allowing organization according to gene ontology (http://www.geneontology.org/). The gene ontology project was originally designed to create a thesaurus-like hierarchy of gene properties, based on molecular function, biological processes and cellular components, in order to integrate databases in a unified way. The combination of raw microarray data and this unified classification will greatly facilitate developing the speculative groundwork for our experiments. This approach differs from the usual methodology based on molecular properties including domain structure, 3D structure, evolution or expression patterns. Therefore, raw microarray data can be a source of new hypotheses, regardless of the original aim of the experiment. This may make it difficult to share raw microarray data. The unified approach, termed MIAME (http://www.mged.org/Workgroups/MIAME/miame.html), for submission of microarray data is described in the guidelines of each paper contributing data to be shared and for peer review. The statement that "MIAME is neither a dogma, nor a legal document-it assumes a cooperative data provider and a fair reviewer" suggests potential difficulty in data sharing, probably stemming from the complexities of ownership and intellectual property rights to data. Microarray data is clearly precious, even in the current era of non-sharing, and should be carefully stored by each lab, organization or government. Such data should be viewed as common property, with potential future benefits to mankind.

FEATURES OF CHOLANGIOCYTES

Bile flows from canalicular spaces, encircled by hepatocytes, through the canals of Hering, interlobular bile ducts (branches of which are 20-100 μm in diameter and lined by cuboidal epithelium), and septal bile ducts (which are more than 100 μm in diameter and lined by a simple tall columnar epithelium) to hilar intrahepatic bile ducts[7]. The cholangiocytes that line the interlobular and bolder bile ducts[7] also contribute to bile secretion[8,9]. The mechanism underlying this phenomenon, described elsewhere in detail, is explained briefly by out-intraluminal vectorial passive transport of water caused by the osmotic gradient which is formed by active transport of several substrates driven by cholangiocytes[10,11]. This physiological function, conserved even in cultured cell lines, characterizes the specialized cholangiocytes. Isolated rat cholangiocytes are classified as large or small, depending on their size. Large cholangiocytes, regarded as representative functional biliary epithelial cells *in vivo*, respond to secretin with increased choleresis[12]. In contrast, little is known about the properties of small cholangiocytes. Cholangiocytes are sometimes altered in a disease-specific manner. For example, primary biliary cirrhosis (PBC), a potentially fatal cholestatic disorder due to ductopenia, is a representative disease characterized by specific destruction of interlobular bile ducts. CD8+ cytotoxic T cells are suspected to play a major role in this type of destruction. Tetramer technology reveals the existence of an E2 subunit of the pyruvate dehydrogenase complex (PDC-E2)$_{159\text{-}167}$-specific to autoreactive cytotoxic T cells in PBC[13]. Hypothetically, there are several specific properties (e.g. adhesion molecules, MHC antigens[14], specific autoantigens, variations in apoptosis and so on) of cholangiocytes lining interlobular bile ducts, which make them vulnerable to attack by cytotoxic lymphocytes. A novel disease etiology was proposed by Gershwin *et al*[15], who demonstrated that IgA class anti-mitochondrial antibodies, transported via transcytosis from the basolateral to the apical surface co-localize with PDC-E2 in cholangiocytes. Moreover, molecules that bind PDC-E2 monoclonal antibody are expressed on the apical membranes of PBC cholangiocytes[15]. These observations indicate the cytotoxicity associated with functional impairment of cholangiocytes to be caused by interaction with IgA-PDC-E2. In this regard, the mechanism of transcytosis may specifically dictate the underlying disease process. Such a hypothesis could be explained by cholangiocyte heterogeneity based on the recognition that large and small cholangiocytes are derived from bile ducts of corresponding diameters[16]. The susceptibility of bile ducts to pathological conditions (e.g. chronic ductopenic rejection[17], GVHD[18], ischemic cholangitis[19], PBC or ductopenia resulting from other pathologies) is due not only to the specific destruction of cholangiocytes, but also to the difficulty in regeneration of the bile ducts i.e. with normal structures, in contrast to the

Table 1 Differences in cDNA expression between small and large cholangiocyte lines

GO[1] annotation/ Gene name	Gene accession	GO accession	Small[2]	Large[3]	Ratio (L/S)
Immune response		GO:0006955			
CD1d1 antigen	NM_007639		107	156	1.46
CD1d2 antigen	NM_007640		80	131	1.64
CD86 antigen; B7-2; CD28 antigen ligand 2 (CD28L2)	L25606		52	17	0.33
Histocompatibility 2, class II antigen E alpha	NM_010381		132	407	3.08
Histocompatibility 2, class II antigen A, beta 1	NM_010379		586	832	1.42
Histocompatibility 2, class II antigen E beta	NM_010382		3579	4481	1.25
Interleukin 2 receptor, beta chain	NM_008368		540	2604	4.83
Cell adhesion		GO:0007155			
Intercellular adhesion molecule 1 (ICAM1)	X52264		39	20	0.51
Vascular cell adhesion molecule 1 (VCAM1)	M84487		17	11	0.65
Cadherin 1 (CDH1); epithelial cadherin (E-cadherin; E-CAD); uvomorulin (UM)	X06115		32	42	1.29
Cytoskeleton		GO:0005856			
Cytoplasmic beta-actin (ACTB)	M12481		455	275	0.60
Vimentin (VIM)	X51438		260	73	0.28
Proteolysis		GO:0006508			
Matrix metalloproteinase 14 (MMP14); membrane-type matrix matalloproteinase 1 (MTMMP1)	X83536		2585	1339	0.52
Cell death		GO:0008219			
Fas antigen ligand (FASL); apoptosis antigen ligand (APTL; APT1LG1); tumor necrosis factor superfamily member 6 (TNFSF6); generalized lymphoproliferative disease protein (GLD)	U06948		37	21	0.57
Fas antigen; fasL receptor; apoptosis antigen 1 (APO1; APT1); CD95 antigen	M83649		18	20	1.07
Fas death domain-associated protein	NM_007829		392	103	0.26
Fas-associating protein with death domain	NM_010175		484	188	0.39
B-cell leukemia/lymphoma protein 2 (BCL2)	M16506		31	18	0.56
Caspase 9	NM_015733		318	996	3.14
Anti-apoptosis					
Insulin-like growth factor 1 receptor alpha subunit (IGFIR-alpha)	U00182	GO:0006916	95	176	1.86
Inflammatory response		GO:0006954			
Tumor necrosis factor	NM_013693		130	275	2.11
Tumor necrosis factor (ligand) superfamily, member 4	NM_009452		98	227	2.32
Tumor necrosis factor (ligand) superfamily, member 7	NM_011617		133	239	1.80
Tumor necrosis factor (ligand) superfamily, member 8	NM_009403		189	514	2.73
Tumor necrosis factor (ligand) superfamily, member 9	NM_009404		278	305	1.10
Tumor necrosis factor (ligand) superfamily, member 19	NM_011615		164	88	0.53
Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A); tumor necrosis factor receptor 1 (TNFR1)	X57796		58	41	0.72
Tumor necrosis factor receptor superfamily member 1B2 (TNFRSF1B2); tumor necrosis factor receptor 2 (TNFR2)	M59378		18	17	0.92
Interleukin 6 receptor alpha subunit (IL6R-alpha; IL6RA)	X51975		57	64	1.13
Cytokine activity		GO:0005125			
Interleukin 6 (IL6)	X06203		36	25	0.70
Oncostatin M (OSM)	D31942		79	509	6.42
Cellular metabolism		GO:0044237			
PDC-E2			NT	NT	
PDC-E3BP			NT	NT	
Transport		GO:0006810			
Solute carrier family 4 (anion exchanger), member 2	NM_009207		80	145	1.82
Solute carrier family 4 (anion exchanger), member 3	NM_009208		52	120	2.31
Solute carrier family 4 (anion exchanger), member 1	NM_011403		995	2146	2.16
Voltage-dependent anion channel 1	NM_011694		111	146	1.32
Voltage-dependent anion channel 2	NM_011695		87	62	0.71
Solute carrier family (organic anion transporter) member 1	NM_013797		76	114	1.50
Aquaporin 1	NM_007472		257	245	0.95
Aquaporin 2	NM_009699		198	276	1.39
Aquaporin 3	NM_016689		124	204	1.64
Aquaporin 5	NM_009701		396	222	0.56
Aquaporin 8	NM_007474		2160	8728	4.04

Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	NM_011387		208	402	1.93
G-protein coupled purinergic receptor P2Y1 (P2RY1)	U22829		346	652	1.89
Purinergic receptor P2X, ligand-gated ion channel 4	NM_011026		711	2229	3.13
Detection of Stimulus		GO0051606			
Toll-like receptor 2	NM_011905		95	68	0.72
Toll-like receptor 5	NM_016928		168	114	0.68
Toll-like receptor 6	NM_011604		255	569	2.23
Cell proliferation		GO:0008283			
FMS-like tyrosine kinase 1 (FLT1); vascular endothelial growth factor receptor 1 (VEGFR1)	L07297		36	23	0.65
Epidermal growth factor (EGF)	J00380		88	116	1.32
Kinase insert domain receptor (KDR); vascular endothelial growth factor receptor 2 (VEGFR2); FLK1	X70842		93	58	0.62
Vascular endothelial growth factor (VEGF); vascular permeability factor (VPF)	M95200		165	152	0.92
Heparin-binding growth factor 5 (HBGF5); fibroblast growth factor 5 (FGF5)	M30643		355	152	0.43
Fibroblast growth factor receptor 3 (FGFR3); heparin-binding growth factor receptor (HBGFR)	M81342		192	485	2.53
Transforming growth factor beta 1 (TGF-beta 1; TGFB1)	M13177		69	105	1.51
Transforming growth factor beta receptor 1 (TGF-beta receptor 1; TGFR1); ESK2	D25540		44	29	0.65
Acetylcholine receptor M3	NM_033269		NT	NT	
Cell cycle		GO:0007049			
Proliferating cell nuclear antigen (PCNA); cyclin	X53068		211	124	0.59
Cell growth					
Estrogen receptor 1 (ESTR1); estrogen receptor alpha (ER-alpha; ESTRA)	M38651	GO:0016049	22	33	1.48
Others					
Nerve growth factor receptor	AF105292	GO:0007411	N.T.	N.T.	
Neurotrophic tyrosine kinase receptor type1	XM283871	GO:0042490	N.T.	N.T.	
Neurotrophic tyrosine kinase receptor type 2	M33385	GO:0042490	93	18	0.20
Neurotrophic tyrosine kinase, receptor, type 3	NM_008746	GO:0042490	2923	3827	1.31
Interferon gamma receptor (IFN-gamma receptor; IFNGR)	M28233	GO:0005615	51	50	0.98

The entire dataset obtained from the Atlas Glass Array mouse (Clontech, Takara Bio Inc., Shiga, Japan) was analyzed by ArrayGauge software (Fuji Photo Film Co., Ltd., Tokyo). [1]gene ontology, [2,3]spot intensities of all samples from small or large cholangiocytes.

rapid regeneration of hepatocytes in cases with acute liver injury. The proliferation of cholangiocytes that potentiate the regeneration of bile ducts occurs in a characteristic manner, *i.e.* secretin, somatostatin or bile duct ligation results in proliferation of large cholangiocytes[20], whereas chemical injury of bile ducts with CCl_4 increases the numbers of small cholangiocytes[21,22]. Therefore, to clarify the mechanisms sustaining cholangiocyte growth it is essential to understand the regulation of bile duct regeneration. We identified an Eph receptor, a membrane bound-type tyrosine kinase, as one of the key molecules for reorganization/proliferation of cholangiocytes, and a subtype of this family, which is expressed mainly in small cholangiocytes[23]. Thus, the heterogeneous expression profile of cholangiocytes is expected to facilitate further study of these cells.

ANALYSIS OF HETEROGENEOUS CHOLANGIOCYTES

For the evaluation of genes that are expressed by small and large cholangiocytes see Table1. Several liver diseases, including non-alcoholic fatty liver disease[24], liver cirrhosis[25], hepatocellular carcinoma[26,27] and cholangiocellular carcinoma[28] have been studied extensively using microarray techniques. Some studies have examined whole liver samples, consisting of various cell types, probably for the purpose of disease classification. Sample size is critical for the detection of subtle changes in the expression of meaningful genes[29], a possible problem in assessing rare diseases. Another problem in the study of biliary diseases like PBC by microarray is that cholangiocytes account for only 3% of the cell population even in the normal liver[30]. Over 75% of the cell population is regarded as necessary to test the significance of differences in expression levels[2]. Therefore, for the purpose of analyzing chlangiocytes, isolation[31] or microdissection[32] is necessary prior to analysis. Our study goals were to characterize the physiological role of biliary epithelia in the mouse and to analyze cholangiocyte heterogeneity. Due to the complexities of the isolation steps, maintaining quality control or reproducibility was possible when obtaining a large sample of RNA from freshly isolated cholangiocytes. Moreover, given the necessity of conducting further functional assays, we immortalized and subcloned the isolated Balb-c mouse cholangiocytes by introducing the SV40 large T antigen[33]. The established large and small cholangiocyte cell lines were evaluated by their morphologies and responsiveness to secretin. We revealed 230 genes (4.74%) showing different expression patterns in the two cells lines, among 4800 genes tested by combining two types of ready-made microarrays[34]. Our large cholangiocyte line was characterized by gene ontology, transport and immune/inflammatory responses, which were apparent, even without the statistical tests presented in the table. The term

"transport" includes movement of anions, water and bile acids, and thus represents the physiological functions of bile ducts. These observations indicate a special feature of large cholangiocytes which presumably play a role in local immune reactions. In contrast, our small cholangiocytes are categorized into a subgroup characterized by rapid cell cycle turnover as well as poor physiological functional ability. In addition to these fundamental properties, our small cholangiocytes are characterized by abundant expressions of actin and vimentin and poor expression of E-cadherin. Together with rich spindle-type cell processes, small cholangiocytes have a feature in common with mesenchymal cells probably originating from epithelial-mesenchymal transition[35]. This remains a controversial issue. Another important feature of cholangiocyte is its capability of the responsiveness to hormones and neuropeptides. Specifically, estrogen receptor[36] and receptor for IGF-1(insulin like growth factor 1)[37], NGF(nerve growth factor)[38] and acetylcholine (M3)[39] have been shown to play a major role in modulating cholangiocyte proliferation. Estrogen receptor expresses at a minimal level in both types of cell lines. In contrast, IGF receptor was preferentially expressed in large cholangiocyte line in our microarray study. The expression of estrogen receptor inducible under pathological conditions like bile duct injury[40] may explain the discrepancy between the experiments results. Predominant expression of IGF receptor in large cholangiocytes may be a marker of differentiated biliary epithelial cells as well as a proliferating effector of maturated cholangiocytes.

CONCLUDING REMARKS

Microarray is a powerful tool for elucidating functional cholangiocyte heterogeneity. Although the evaluation of some crucial biological regulatory processes like protein modification requires methodologies other than microarray, the potential of microarray technology is anticipated to grow with the development of data-analysis theory for the comprehension of complex networks.

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Ursodeoxycholic acid treatment of vanishing bile duct syndromes

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Abstract

Vanishing bile duct syndromes (VBDS) are characterized by progressive loss of small intrahepatic ducts caused by a variety of different diseases leading to chronic cholestasis, cirrhosis, and premature death from liver failure. The majority of adult patients with VBDS suffer from primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). Ursodeoxycholic acid (UDCA), a hydrophilic dihydroxy bile acid, is the only drug currently approved for the treatment of patients with PBC, and anticholestatic effects have been reported for several other cholestatic syndromes. Several potential mechanisms of action of UDCA have been proposed including stimulation of hepatobiliary secretion, inhibition of apoptosis and protection of cholangiocytes against toxic effects of hydrophobic bile acids.

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Key words: Cholestasis; Primary biliary cirrhosis; Primary sclerosing cholangitis; Secretion; Signaling; Transport; Ursodeoxycholic acid; Vanishing bile duct syndrome

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INTRODUCTION

Cholestasis is defined as an impairment of bile flow and failure to adequately secrete cholephilic compounds into bile often associated with clinical manifestations such as jaundice and pruritus and biochemical alterations such as an elevation in serum alkaline phosphatase and γ-glutamyl transpeptidase. The liver damage observed in chronic cholestasis has long been attributed to accumulation and retention of potentially toxic bile acids within hepatocytes. Chronic cholestasis is the main feature of vanishing bile duct syndromes (VBDS) characterized by progressive loss of small intrahepatic ducts caused by a variety of different diseases. In children, biliary atresia and Alagille syndrome are rare ductopenic diseases classified as VBDS. The majority of adult patients with VBDS suffer from primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). Other diseases associated with ductopenia include autoimmune cholangitis, chronic hepatic allograft rejection, graft-versus-host disease (GVHD), chronic cholestatic sarcoidosis and ischemic (vascular) cholangiopathies. Among the various causes of VBDS, drugs have an increasing importance. Drug reactions are related especially to antibiotics, phenothiazine derivates and carbamazepine. It is increasingly clear that immunopathogenetic mechanisms involving innate and adaptive immune responses contribute to ductopenia in most of these diseases. Prognosis varies from hepatic failure and death or liver transplantation to resolution of cholestasis and normal liver function.

Ursodeoxycholic acid (UDCA) is a hydrophilic dihydroxy bile acid (chemical structure: 3α, 7β-dihydroxy-5β-cholanoic acid) which was first identified in the bile of the Chinese black bear[1]. UDCA is a physiologic bile acid also present in man albeit in a low concentration of about 3% of the bile acid pool, where it is formed by 7β-epimerization of the primary bile acid chenodeoxycholic acid in the gut by intestinal bacteria[2,3]. It has been used for centuries in traditional Chinese medicine for the treatment of liver diseases. Reports from Japan and Europe first revealed that UDCA was able to dissolve gallstones[4-6]. In 1985, Leuschner and coworkers observed improved serum liver tests in patients with chronic active hepatitis treated with UDCA for gallstone dissolution[7], and similar observations were made previously in Japan. Since then, various trials have shown the beneficial effect of UDCA for different cholestatic syndromes.

PHARMACOKINETICS AND METABOLISM OF UDCA

After oral administration, UDCA is absorbed by passive nonionic diffusion mainly in the small intestine (-80%)

and less in the colon (-20%) following solubilization in mixed micelles of endogenous bile acids in the proximal jejunum[2,8]. Its absorption rate is enhanced when it is given with a meal and may be decreased in patients with cholestasis and decreased biliary secretion of endogenous bile acids[9]. After intestinal absorption, UDCA is taken up from the portal blood by the hepatocytes at their sinusoidal domain *via* specific bile acid transporters namely, NTCP and OATP, conjugated mainly with glycine and to a lesser extent with taurine and is subsequently transported across the canalicular domain into the bile ducts *via* another bile acid carrier molecule, designated BSEP[2,10]. UDCA conjugates reach the small intestine and are reabsorbed mainly from the distal ileum *via* an active Na^+-dependent transport mechanism undergoing an effective enterohepatic circulation. Non-absorbed UDCA and UDCA conjugates pass into the colon and, after bacterial deamidation of conjugates, are mostly converted to lithocholic acid by intestinal bacteria and eliminated *via* the feces[2]. Only minute amounts of the insoluble lithocholic acid are reabsorbed *via* the colonic mucosa, sulphated in the liver, secreted into bile and excreted in the feces. Under continuous oral treatment at pharmacological doses (13-15 mg/kg per day) UDCA becomes the predominant bile acid in the liver and the systemic circulation, comprising 40% to 60% of total bile acid[2,8].

MECHANISMS OF ACTION OF URSODEOXYCHOLIC ACID

The mechanisms underlying the beneficial effects of UDCA in cholestasis are being increasingly unraveled[11-14]. Initial research interest was focused on changes in bile acid pool composition, hepatocyte membrane protective effects, immunomodulatory effects, and bicarbonate-rich hypercholeresis induced by UDCA. Over the past decade, it has, however, become evident that UDCA is a potent intracellular signaling agent that induces stimulation of impaired hepatocellular secretion, anti-apoptotic effects and may mediate cholangiocyte protection. Depending on the pathophysiology and the stage of the underlying liver disease, the predominant mechanisms of action of UDCA may vary.

Stimulation of hepatobiliary secretion

Cholestatic liver diseases are characterized by an impairment of hepatobiliary secretion. As a consequence, bile acids and other potentially toxic cholephiles accumulate in the hepatocyte and may lead to liver cell injury, apoptosis and necrosis. UDCA stimulates biliary secretion of bile acids and other organic compounds (e.g. bilirubin glucuronides, glutathione conjugates, bromosulfophthalein) in various experimental models such as the isolated hepatocytes, isolated perfused rat liver and bile fistula rat model and counteracts cholestasis induced by hydrophobic bile acids in rat liver[15-22]. In line with these observations, biliary secretion of bile acids and phospholipids is stimulated and elevated serum levels of the hydrophobic bile acid, chenodeoxycholic acid, and of bilirubin are decreased in patients with PBC and PSC during UDCA treatment[23-28]. Thus, the beneficial effects of UDCA in cholestatic liver disease may be partly due to the enhanced elimination of toxic compounds from the hepatocytes. The secretory capacity of the hepatocytes is determined by the number and activity of transporter proteins in the canalicular membrane. UDCA stimulates the expression of transporter proteins for biliary secretion in the liver and the targeting and insertion of transporter molecules into the canalicular membrane at a transcriptional and posttranscriptional level[15,29-31]. As such, UDCA stimulates the overall gene expression of both canalicular (Mrp2, Bsep) and alternative basolateral carriers (Mrp3, Mrp4) in mouse liver, which facilitates alternative efflux of bile salts and other organic anions into the systemic circulation[32,33]. UDCA also stimulates murine renal (Mrp2, Mrp4) and intestinal (Mrp2, Mrp3) efflux transport proteins, resulting in an increased overall elimination capacity for potentially toxic biliary compounds[32]. In addition to these transcriptional effects, UDCA also stimulates vesicular exocytosis and insertion of transporter proteins into the canalicular membrane by modulating complex intracellular signalling cascades including calcium, protein kinase C and different mitogen-activated protein kinases[15,31,34-36]. Moreover, UDCA may also directly activate canalicular transporters through modification of their phosphorylation status[37]. While effects of UDCA on mRNA and protein levels of transporters may be important for long-term regulation, the effects on the insertion into the canalicular membrane and the activity of transporters may determine short-term regulation of secretion. In conclusion, UDCA modulates hepatobiliary secretion by transcriptional and posttranscriptional mechanisms in experimental models *in vivo* and *in vitro*. Thus, upregulation of synthesis, apical targeting and insertion, and activation of key canalicular transporters may represent key mechanisms to explain the anticholestatic action of UDCA in patients with cholestatic liver disease.

Inhibition of apoptosis

Apoptosis, or programmed cell death, is an important mechanism of cell death in cholestatic liver diseases[14,38,39]. For example, apoptotic features causing bile duct loss have been observed more frequently in liver tissue from patients with PBC than in normal controls[40]. Toxic bile acids can induce apoptosis in hepatocytes at concentrations comparable to those found in chronic cholestasis and the mechanisms of bile acid-induced apoptosis have increasingly been understood (reviewed by Higuchi *et al*[41]). Endogenous hydrophobic bile acids such as glycochenodeoxycholic acid (GCDCA) or glycodeoxycholic acid (GDCA) induce apoptosis by ligand-independent activation of the Fas death-receptor, followed by activation of caspase 8 and Bid, a pro-apoptotic member of the Bcl-2 protein family, which chaperones another pro-apototic Bcl-2 molecule, Bax, to the mitochondrial membrane inducing mitochondrial membrane permeability transition (MMPT). MMPT causes a sudden increase in permeability of the inner mitochondrial membrane to ions followed by

mitochondrial swelling and release of cytochrome c from the intermembrane space to the cytosol. In the cytosol, cytochrome c interacts with the apoptotic protease-activating factor 1 (APAF-1), which leads to activation of caspase 9 and subsequently causes apoptotic cell death[40,42].

A number of studies showed that UDCA can block apoptosis *in vitro* as well as *in vivo* in the rat and in human hepatocytes by interrupting classic pathways of apoptosis[43-45]. Antiapoptotic effects of UDCA were associated with a reduction of the MMPT and mitochondrial cytochrome c release[14,45]. UDCA protects rat hepatocytes against bile acid-induced apoptosis by preventing bile acid-induced, c-Jun N-terminal kinase-dependent Fas trafficking to the plasma membrane[46]. Apoptosis can not only be inhibited by blocking pro-apoptotic pathways, but also by activation of intracellular survival signals. Indeed, UDCA was shown to induce a survival signal in hepatocytes *via* activation of the epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) that may contribute to the antiapoptotic effect[47]. Thus, UDCA appears to be a bile acid that partially activates pro-apoptotic signals, but also simultaneously a strong MAPK-dependent survival signal, which makes UDCA an overall non-toxic bile acid. Although intriguing, the impact of antiapoptotic mechanisms for the beneficial effects of UDCA in cholestatic liver disease remains unclear at present.

Protection of cholangiocytes against toxic effects of hydrophobic bile acids

Hydrophobic bile acids damage cell membranes of hepatocytes and cholangiocytes and exert extracellular cytotoxicity at millimolar concentrations present in bile[48-50]. Indeed, levels of hydrophobic bile acids are about 1000-fold higher in bile than in serum even under physiological conditions. The proposed mechanisms of bile acid-induced cell damage extend from simply binding to plasma membranes to the induction of apoptosis or even necrosis. UDCA has been shown to counteract hydrophobic bile acid-induced membrane disruption *in vitro* presumably by alteration of the structure and composition of simple and mixed micelles rather than by direct membrane interactions[50,51]. UDCA treatment at therapeutic doses of 13-15 mg/kg per day renders the bile acid composition of bile less hydrophobic leading to a relative enrichment of UDCA to about 40%-50% of total biliary bile acids[8]. To achieve this effect in isolated cells or whole organ a high (millimolar) concentration of UDCA is required. Thus, the membrane protective effects of UDCA play a role mainly at the bile duct level, since high concentrations of bile acids are only present within the biliary tree[52]. UDCA decreases the degree of cholangiocellular injury, portal inflammation and ductular proliferation in the Mdr2-knockout mouse, an animal model of cholestasis which shares morphological features with PSC resulting from the toxic effect of bile acids on the biliary epithelium in the absence of protective mixed micelle formation with phospholipids[53,54]. In line with these observations, the (peri-) portal inflammatory reaction is less severe in patients with PBC and PSC under UDCA treatment as compared to those treated with placebo[27,28,55,56]. The effects of UDCA conjugates on cholangiocytes are apparently mediated by Ca^{2+}- and protein kinase Cα-dependent mechanisms which have been implicated in stimulation of biliary secretion in cholestatic hepatocytes as outlined above. Although the protection against the consequences of bile duct destruction is likely to be one mechanism of action of UDCA, the underlying pathophysiologic events leading to the ongoing bile duct destruction are probably not influenced by UDCA.

UDCA IN VANISHING BILE DUCT SYNDROMES

Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by chronic inflammation and destruction of intrahepatic bile ductules, which leads to progressive ductopenia. ultimately resulting in fibrosis and biliary cirrhosis. The prevalence differs considerably in different geographic areas, ranging from 40 to 400 per million. It primarily affects women, with a female to male ratio as high as 10 to 1[57,58]. Current theories on the pathogenesis of PBC favor the hypothesis that the disease develops as a result of an inappropriate immune response following stimulation by an environmental or infectious agent. The pathogenetic mechanism is believed to be caused by a defect in immunologic tolerance, resulting in the activation and expansion of self-antigen specific T and B lymphocyte clones and the production of circulating autoantibodies in addition to a myriad of cytokines and other inflammatory mediators. The serologic hallmark of PBC is the presence of autoantibodies to mitochondria, especially to the E2 component of the pyruvate dehydrogenase complex (PDC)[59]. 50% to 60% of patients are asymptomatic at diagnosis, and the disease is initially detected on the basis of screening liver-biochemistry profiles. Fatigue and pruritus are the most common presenting symptoms[60], occurring in up to 78% and 70% of patients, respectively. Jaundice develops usually later, often associated with progression of the disease[61]. Eventually, complications of cirrhosis and portal hypertension, such as ascites, variceal bleeding, and hepatic encephalopathy, develop.

UDCA is now the mainstay of therapy for PBC. In 1987 Poupon and co-workers reported benefit from UDCA treatment[62]. Since then, several randomized double-blind placebo-controlled studies have shown that UDCA improves biochemical parameters including serum bilirubin levels which are used as a prognostic marker for PBC[25,27,55,63-66]. Indeed, it has been shown that the mean survival in patients with serum bilirubin levels above 34 μmol/L is 4 years; in those with values of more than 103 μmol/L only 2.1 years[67]. This prognostic value of serum bilirubin is similar in UDCA-treated patients as in non-treated patients. The benefit from UDCA therapy on liver fibrosis progression in PBC has been shown by Corpechot *et al*[68] using Markov modeling: A fivefold decrease in progression rate from early-stage disease toward extensive fibrosis or cirrhosis was found in UDCA-treated

patients compared with placebo-treated patients. At 4 years, the probability of UDCA-treated patients to remain in early stage disease was 76% (95% confidence interval: 58%-88%), as compared with 29% (15%-52%) in placebo-treated patients. These findings are consistent with recent data showing that a 2-year treatment with UDCA reduces periportal necroinflammatory lesions, improves ductular proliferation, and delays the progression of histologic stage when given at the earlier stages (stages I-II) of the disease[69]. When started later in the disease course, UDCA can still ameliorate inflammation and ductular proliferation but is not capable of reversing fibrosis.

What is still more debatable is the effect of UDCA treatment on long-term survival. A combined analysis of three larger randomized trials, in which patients with primary biliary cirrhosis were randomly assigned to receive UDCA (n = 273) or placebo (n = 275) suggested that survival free of liver transplantation was significantly improved in patients who received UDCA treatment for 4 years[70]. This benefit was seen in patients with moderate to severe disease but not in those with mild disease (serum bilirubin concentration < 23.9 μmol/L or histologic stage I or II) probably because progression to end-stage disease did not occur in the short time interval of the study in patients with mild disease[70]. Lindor *et al* showed that survival of patients treated up to 6 years with UDCA was increased as compared with a placebo group or the predicted survival from the Mayo model[71]. In a large cohort of UDCA-treated PBC patients (225 patients) followed for up to 10 years survival free of liver transplantation was significantly higher than survival predicted by the Mayo model and 10-year survival among UDCA-treated patients is slightly lower than that of an age- and sex-matched population, the difference being mainly explained by mortality among cirrhotic patients[72]. However, the efficacy of UDCA in PBC has also been questioned. The extended follow-up of another clinical trial[73], as well as a randomized controlled trial with the longest follow-up[55], did not show a favorable effect of UDCA on the incidence of death or liver transplantation. In addition, a systematic review and meta-analysis of 11 randomized controlled trials, including 1272 patients, and six reports of the switch-over phases did not find a beneficial effect of UDCA on the incidence of liver-related death, liver transplantation, or the development of complications of liver disease[74]. Similarly, another meta-analysis of 16 randomized clinical trials evaluating UDCA against placebo (n = 15) or no intervention (n = 1) in 1422 patients did not detect a significant effect of UDCA on mortality[75]. However, these meta-analyses included trials of different length of follow-up, mostly only up to 24 mo, which were performed with various doses of UDCA, in part less than 13-15 mg/kg per day, a dose currently regarded as optimal for PBC. Thus, these meta-analyses have to be interpreted with caution, because survival analyses in a disease with a very long natural history over decades are ideally based on longer follow-up periods. In a further analysis including five studies with a follow-up of at least 4 years, a 32% reduction in the risk of death or need of liver transplantation was reported in patients treated with UDCA[76]. Nonetheless, to clarify the true efficacy of UDCA in the long-term treatment of PBC, additional data are needed. At present, the general recommendation is to treat PBC patients with UDCA at a dose of 13-15 mg/kg per day. Treatment should be started as early as possible, since patients with mild or moderate disease are likely to have a greater benefit from therapy than those with advanced disease.

Primary sclerosing cholangitis (PSC)

Primary sclerosing cholangitis (PSC) is a rare chronic cholestatic disease of the intra- and extrahepatic bile ducts that is generally progressive and leads to end-stage liver disease with a median survival from diagnosis to death or liver transplantation, currently estimated at 12 years[77-80]. PSC is characterized by diffuse inflammation, concentric obliterative fibrosis and progressive stricturing and dilatation of the biliary tree. The etiology and pathogenesis is unknown, but as in PBC, immune dysregulation plays an important role in the development of the disease. In Western populations, the estimated prevalence of PSC is 8.5 cases per 100 000 persons[81] and 70%-80% of PSC patients have or will develop inflammatory bowel disease. Of importance, patients with PSC have an increased risk of developing cholangiocarcinoma with an estimated yearly incidence of 1.5% after diagnosis of PSC[82-85] and patients with both PSC and ulcerative colitis may be at higher risk for developing colorectal aneuploidy, dysplasia, or cancer than UC patients without PSC[86,87]. Based on the beneficial effect of UDCA in patients with PBC, UDCA was evaluated in a number of small trials for patients with PSC in the early 1990s[28,88,89]. Although small, these studies observed significant biochemical and histologic improvements for patients receiving UDCA. Similar to the treatment of PBC, no major side effects were reported. In contrast to these promising results, the first large randomized placebo-controlled study including 105 patients with PSC could not find a clinical benefit of UDCA treatment with respect to time to treatment failure or histologic findings in a dose of 13-15 mg/kg per day during a mean follow-up period of 2.2 years[26]. A meta-analysis of six randomized clinical trials showed no difference between UDCA and placebo in the effects of incidence on death, treatment failure (including liver transplantation, varices, ascites, and encephalopathy), liver histological deterioration or liver cholangiographic deterioration[90]. However, most trials were small with an average sample size of 37 patients and the follow-up period might have been too short to show a clinical benefit, since PSC has usually a long natural history of over a decade. A large percentage of the patients had advanced disease and as in PBC, these patients might respond less to medical treatment than patients in earlier stages of disease. Finally, UDCA therapy alone will not suffice to treat the bile duct strictures typical for PSC, but may need additional mechanical intervention. Indeed, major bile duct strictures develop during UDCA treatment[91]. Based on these observation, UDCA treatment was combined with endoscopic dilatation of bile duct strictures in an 8-year prospective study. The survival of patients receiving this combination therapy was significantly better than the calculated survival without treatment[92]. As biliary enrichment of UDCA is expected to be lower in cholestasis and because of discouraging results with standard doses of UDCA (13 -15

mg/kg per day), use of high doses of UDCA in PSC has a rationale and has been evaluated by three groups[93-95]. The first study compared high-dose UDCA (20 mg/kg per day) (n = 13) to placebo (n = 13) for 2 years in 26 patients with PSC and demonstrated a significant improvement in serum levels of AP and γ-GT (no effect on bilirubin and albumin levels), and a significant reduction in progression of cholangiographic features and liver fibrosis as assessed by disease staging without significant side effects. UDCA did not improve symptoms[94]. Harnois *et al*[93] reported similar results in a 1-year open-label study of 30 PSC patients treated with UDCA at a dose of 25-30 mg/kg per day. Changes in the Mayo risk score at 1 year of treatment and projected survival at 4 year were compared with that observed in patients randomized to placebo or UDCA at a dose of 13-15 mg/kg per day in a previous study. A significant improvement in serum alkaline phosphatase activity, AST, albumin and total bilirubin occurred at 1 year of therapy with high-dose UDCA. The expected survival at 4 years was significantly improved for patients in the high-UDCA group when compared with a historical placebo control, but not between the dose of 13-15 mg/kg per day and placebo. As in the first study, high-dose UDCA was well tolerated. Finally, in the biggest placebo-controlled prospective study in PSC ever performed, a total of 219 patients were randomized to 17 to 23 mg/kg per day of UDCA (n = 110) or placebo (n = 109) for 5 years[95]. No statistically significant benefit from 20 mg/kg UDCA on survival without liver transplantation or prevention in cholangiocarcinoma in PSC was shown in this study. However, there was a tendency to improved survival in the UDCA-treated patients and the study was underpowered to show a possible positive effect.

UDCA has also been shown to have colon cancer chemopreventive effects in preclinical studies[96,97]. In addition, two clinical studies suggested that UDCA also decreases the risk for developing colorectal neoplasia in UC patients with PSC[98,99]. In a cross-sectional study by Tung *et al* involving 59 patients with ulcerative colitis and primary sclerosing cholangitis who were undergoing colonoscopic surveillance for colonic dysplasia, UDCA use was strongly associated with decreased prevalence of colonic dysplasia (odds ratio, 0.18, 95% confidence interval, 0.05 to 0.61; P = 0.005)[98]. In a blinded, prospective study by Pardi *et al*[99], 52 patients with ulcerative colitis and PSC were randomized to receive UDCA or placebo and were followed-up for a total of 355 person-years. Those originally assigned to receive UDCA had a relative risk of 0.26 for the development of dysplasia or cancer (95% confidence interval, 0.06-0.92; P = 0.034) suggesting a significant chemoprotective effect for UDCA in these patients.

In conclusion, UDCA therapy (13-20 mg/kg per day) of PSC is warranted and should be initiated as soon as possible and be continued life-long. In addition, major strictures should be dilated regularly in centers with experienced endoscopists. In patients with PSC and ulcerative colitis, who carry a considerable lifetime risk for colorectal neoplasia, UDCA may act as a chemopreventive drug. Due to the unresolved issues of optimal dosing and the true long-term benefit of UDCA in PSC, patients should be included in trials if possible.

Intrahepatic cholestasis of pregnancy

Intrahepatic cholestasis of pregnancy (ICP) is the most common pregnancy-related liver disorder characterized by pruritus associated with a mild or moderate increase in serum aminotransferases and serum bile acids starting in the second or third trimester of pregnancy and disappearing after delivery. ICP is associated with increased risk of intrauterine fetal death and premature delivery[100,101]. There is increasing evidence that genetically determined dysfunction in the canalicular ABC transporters bile salt export pump and multidrug resistance protein 3 might be risk factors for ICP development[102]. In small controlled trials and several observational trials, UDCA improved maternal pruritus and serum liver chemistries such as serum bilirubin and transaminases, and diminished the number of premature deliveries. The medication seems to be well tolerated by pregnant women and no adverse effects in mothers or newborns have been detected[103-105]. A recent prospective, randomized trial (n = 84) comparing cholestyramine (8 g daily) and UDCA (8-10 mg/kg daily) for the treatment of ICP further supported these findings. Pruritus was more effectively reduced by UDCA than cholestyramine. Newborns were delivered significantly closer to term by patients treated with UDCA, than those treated with cholestyramine. Serum alanine and aspartate aminotransferase activities were markedly reduced by 78.5% and 73.8%, respectively, after ursodeoxycholic acid, but by only 21.4%, each, after cholestyramine therapy. Ursodeoxycholic acid, but not cholestyramine was free of adverse effects[106]. In ICP, the relief of cholestasis by UDCA has been suggested to be due to stimulation of vesicular exocytosis resulting in mobilization of an increased number of transport proteins to the canalicular membrane and, thereby, stimulation of transport systems involved in the biliary secretion of steroid mono- and disulfates. In conclusion, data that are available support the use of UDCA as first-line therapy for ICP in the third trimester.

Hepatic complications of allogeneic bone marrow transplantation

Hepatic graft-versus-host disease (GVHD) is a frequent complication after bone-marrow transplantation usually associated with a cholestatic picture characterized by elevated serum alkaline phosphatase and hyperbilirubinemia attributed to damage of the small bile ducts. UDCA has been reported to be effective in the treatment of manifest GVHD[107,108]. In an open-label study 12 patients with refractory chronic GVHD of the liver after allogeneic bone marrow transplantation were given UDCA (10 to 15 mg/kg per day) for 6 wk. Serum tests of cholestatic liver injury showed improvement compared with baseline. After discontinuation of UDCA therapy, 11 patients were followed for 6 additional weeks. All showed significant worsening in liver function test results. Symptom scores were unchanged throughout the study. No adverse effects were observed[107]. In addition, the efficacy of UDCA has also been reported as a prophylaxis for veno-occlusive disease (VOD) of the liver after

allogeneic bone marrow transplantation. In a randomized, double-blind, placebo-controlled study including 67 consecutive patients undergoing transplantation with allogeneic bone marrow with a preoperative regimen of busulfan plus cyclophosphamide were randomly assigned to receive UDCA 300 mg twice daily or placebo. The incidence of VOD was 40% in placebo recipients and 15% in the UDCA-treated recipients ($P = 0.03$)[109]. Thus, further studies are certainly needed, but UDCA may be considered for prophylaxis of VOD and treatment of GVHD of the liver.

Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) gene resulting in the secretion of viscous bile. This may lead to the formation of bile duct plugs within small bile ducts, biliary obstruction and ultimately result in (focal to multilobular) biliary cirrhosis. Hepatobiliary complications increase with patient age and up to 7% of children and young adults with CF may present with liver cirrhosis[110]. Early uncontrolled studies showed that UDCA significantly improved laboratory tests and nutritional status of patients with CF[111-113]. A small randomized, placebo-controlled study showed clinical, biochemical and nutritional improvement in CF patients treated with UDCA (15 mg/kg per day) for one year[114]. Improvement of liver histology of CF patients treated with UDCA (10-15 mg/kg per day) for 2 years has been reported as well[115]. A higher dose of 20 mg/kg per day may be more efficious than lower doses (5-15 mg/kg per day)[111,116]. Thus, UDCA may be considered as potentially effective in CF patients. However, whether UDCA affects the natural history of liver disease in CF-particularly amelioration of the complications of portal hypertension, need for liver transplantation, or a measurable survival is unknown and further well-designed, adequately powered, randomized controlled trials should address these issues. Stimulation of biliary secretion may be the major mechanism for the therapeutic effect. Interestingly, it has been hypothesized that the therapeutic effect of UDCA is mediated in part by stimulation of ATP release into bile and subsequent ATP-induced bicarbonate secretion by bile-duct epithelia *via* calcium-dependent chloride channels distinct from the CFTR[117].

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Cholangiocyte anion exchange and biliary bicarbonate excretion

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Abstract

Primary canalicular bile undergoes a process of fluidization and alkalinization along the biliary tract that is influenced by several factors including hormones, innervation/neuropeptides, and biliary constituents. The excretion of bicarbonate at both the canaliculi and the bile ducts is an important contributor to the generation of the so-called bile-salt independent flow. Bicarbonate is secreted from hepatocytes and cholangiocytes through parallel mechanisms which involve chloride efflux through activation of Cl^- channels, and further bicarbonate secretion *via* AE2/SLC4A2-mediated Cl^-/HCO_3^- exchange. Glucagon and secretin are two relevant hormones which seem to act very similarly in their target cells (hepatocytes for the former and cholangiocytes for the latter). These hormones interact with their specific G protein-coupled receptors, causing increases in intracellular levels of cAMP and activation of cAMP-dependent Cl^- and HCO_3^- secretory mechanisms. Both hepatocytes and cholangiocytes appear to have cAMP-responsive intracellular vesicles in which AE2/SLC4A2 colocalizes with cell specific Cl^- channels (CFTR in cholangiocytes and not yet determined in hepatocytes) and aquaporins (AQP8 in hepatocytes and AQP1 in cholangiocytes). cAMP-induced coordinated trafficking of these vesicles to either canalicular or cholangiocyte lumenal membranes and further exocytosis results in increased osmotic forces and passive movement of water with net bicarbonate-rich hydrocholeresis.

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Key words: AE2 anion exchanger; Bile salt-independent flow; Biliary bicarbonate excretion; Regulation of intracellular pH; Hydroionic fluxes in cholangiocytes

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INTRODUCTION

Hepatocytes and cholangiocytes are the epithelial cells in the liver, and they both participate in the production of bile. The hepatocytes, the major liver cell population (65%), generate the primary bile at their canaliculi[1]. As an important complement, the epithelial cells lining intrahepatic bile ducts or cholangiocytes (5% of the liver cell population) exert a series of reabsorptive and secretory processes which dilute and alkalinize the bile flow during its passage along the biliary tract[2-6]. Modifications of ductal bile appear to be tightly regulated by the action of nerves, biliary constituents, and some peptide hormones like secretin. Accordingly, it is possible to distinguish between three different bile flow fractions: (1) the canalicular bile salt-dependent flow (30% to 60% of spontaneous basal bile flow) that is driven by concentrative secretion of bile acids by the hepatocytes followed by a facilitated efflux of water[7,8]; (2) the canalicular bile salt-independent flow (another 30% to 60% of spontaneous basal bile flow)[1,8-10], which is also created by hepatocytes but through active secretion of both inorganic and organic compounds (mainly bicarbonate[11,12] and glutathione[13], respectively); and (3) the ductal bile flow, that is the bile salt-independent flow fraction modified and contributed by cholangiocytes, mainly through production of a bicarbonate-rich fluid in response to secretin[2,14-17] and other regulatory factors[2,5,6,17,18].

In the last decade, the use of molecular- and cell-biology tools and the availability of suitable experimental models have greatly facilitated our knowledge on the processes involved in bile generation and modification. This review summarizes some of the experimental models employed in biliary studies, and focuses on the biliary excretion of bicarbonate, the chief factor responsible for ductal bile alkalinization and fluidization, and the role and interactions of regulatory factors.

STUDY MODELS

Biliary studies started over one hundred years ago. In 1902 Bayliss and Starling discovered the hormone secretin as an agent with stimulatory effects on pancreatic secretion and bile flow[19]. About 25 years later secretin was reported as a cholagogue, i.e. an agent capable of stimulating the flow of bile into the duodenum[20], as well as a choleretic agent that could stimulate the production of bile in the liver[21]. In spite of this, although the role of hepatocytes for bile generation was widely recognized quite early, the contribution of cholangiocytes to the production of bile with an adequate composition was accepted more recently. Knowledge of the cholangiocyte contribution was facilitated by the availability of experimental models and the development of both *in vivo* and *in vitro* sophisticated procedures.

In vivo models

The initial experimental studies on the bile flow and bile composition were carried out by using *in vivo* models of conscious or anesthetized mammals with biliary fistulas. In the case of total biliary fistulas, there was a major concern because of the interruption of the enterohepatic circulation and the subsequent depletion of the bile acid pool. However, this can be overcome by continuous intravenous or intraduodenal administration of exogenous bile acids at controlled rates. On the other hand, partial biliary fistula allows controlled interruption of the enterohepatic circulation, and a portion of bile collected can be returned to the stomach or duodenum, thereby replenishing the pool of natural bile acids[10]. Fistula animals have also been useful to estimate canalicular bile flow by measuring the biliary clearance of selected inert solutes (mainly erythritol and mannitol). This procedure assumes that these solutes are sufficiently permeable to enter the canalicular bile by passive processes while being unable to cross the ductal epithelium[22,23]. It appears that this might be the case in some animal species but not in all[24]. Moreover, bile duct cannulation allows direct interventions in the biliary tract through retrograde intrabiliary injection[25-28]; this procedure has been employed to obtain animal models of hepatitis[29] and toxic-induced biliary disease[30], as well as for experimental gene therapy[31,32]. Retrograde intrabiliary injection has also been employed to assess the effects of toxic substances or inhibitors on the bicarbonate-rich choleresis upon stimulation. For instance, ursodeoxycholate-induced bicarbonate-rich choleresis has been shown to be sensitive to intrabiliary phenol[33]. More recently, secretion of bicarbonate in secretin-stimulated rats has been found to be sensitive to intrabiliary administration of particular ion-transport blockers[17].

In humans, *in vivo* assessment of biliary bicarbonate secretion has employed cumbersome maneuvers like nasobiliary drains in hepatic bile ducts[34]. In a context of surgical interventions, invasive procedures similar to those employed in animals (for instance T-tube insertion into the common bile duct[35,36] or percutaneous transhepatic cholangio-drainage[37]) were also used. Recently, non-invasive assessment of biliary bicarbonate secretion was developed by using positron emission tomography (PET)[38]. This imaging technique allows evaluation of baseline and stimulated organ functions after intravenous injection of short half-live positron emitting isotopes[39,40]. Thus, 2-3 min after bicarbonate labeled with carbon-11 (half live of 20.4 min) was given to healthy volunteers, label uptake was observed in the abdominal region corresponding to the liver parenchyma and hepatic hilum. Interestingly, administration of secretin increased bicarbonate uptake in the parenchymal region, this being followed by accumulation of the label in the perihilar area[38]. Currently, the availability of micro-PET systems may also facilitate non-invasive *in vivo* studies of biliary bicarbonate secretion in small laboratory animals.

Animal models of bile ductal cell hyperplasia have also been developed, mainly in rodents, to study the pathophysiology of bile ducts[41-43]. These models are closely associated with increased secretin-stimulated ductal secretion. The increased responsiveness seems to occur regardless of the procedure employed to obtain the hyperplasia of cholangiocytes: partial hepatectomy[44], chronic feeding of bile acids[45] or α-naphthylisothiocyanate (ANIT)[46], chronic administration of phenobarbital plus CCl_4 to rats[47] or just CCl_4 in mice[48], or bile-duct ligation (BDL)[41,49]. In contrast, a decreased secretin-stimulated ductal secretion can be observed in BDL rats with ductopenia following interruption of the cholinergic innervation by total vagotomy[50], or selective damage of large (but not small) cholangiocytes by acute feeding with CCl_4[51,52].

The development of genetically modified murine models has contributed to ascertain the role of selective genes, like those for the cystic fibrosis transmembrane conductance regulator *Cftr*[53,54] and the P-glycoprotein PGY3/MDR2-3/ABCB4 *Mdr2*/*Abcb4*[55], among others. In *Cftr*$^{-/-}$ mice, for instance, induction of colitis has been shown to result in increased bile duct injury[53]. In *Mdr2*/*Abcb4*$^{-/-}$ mice, a multistep process of bile-duct damage leading to sclerosing cholangitis has been described[55]. Spontaneous mutant animals can also serve as useful *in vivo* models. The PCK rat, a model of the autosomal recessive polycystic kidney and hepatic disease (PKHD), has been used for studies on possible trigger factors of biliary cystogenesis. This mutant was spontaneously developed in the rat strain Crj:CD/Sprague-Dawley because of a germ line mutation of the *Pkhd1* gene[56,57]. The TR$^-$ rat model is widely used for studying canalicular secretion of organic anions. This mutant lacks the functional canalicular isoform of the conjugate export pump MRP2/ABCC2 because of one-nucleotide deletion in the *Mrp2*/*Abcc2* gene[58].

In vitro models

Isolated perfused liver preparations are useful experimental models to evaluate the liver effects of single factors in a manner independent of systemic or humoral effects[59]. There are two modalities of these preparations, as the liver can be isolated and perfused *in situ* or *ex situ*, i.e. attached to or removed from the animal body. Livers can be isolated from many animal species (rat[1,59], hamster[60], guinea pig[61], cat[62], rabbit[63], dog[64], sheep[65], calf[66], pig[67], and

monkey[68]), the isolated perfused rat liver (IPRL) being the model most widely used[69]. All these models allow repeated collection of both the perfusate and the bile, and permit easy exposure of the liver to different concentrations of test substances. Test substances may be given intravascularly through just the portal vein[1,59-61,70], which provides flow to essentially all hepatocytes[71], or through both the portal vein and the hepatic artery (i.e. isolated bivascularly perfused liver)[72,73]. The latter bivascularly perfused model is particularly adequate to investigate ductal physiology because the predominant blood supply to the bile ducts is *via* the hepatic artery[74,75]. For studies on ductal secretion, test substances and drugs may also be administered intrabiliary (as retrograde intrabiliary injection[76]). Altogether, these *in vitro* preparations of isolated perfused liver had provided very valuable data on liver physiology and pathophysiology, the regulation of bile secretion included. However, extrapolation of that data to the *in vivo* situation should be cautious, as *in vivo* effects of local factors may be tightly influenced by systemic players such as humoral factors and innervation.

Membrane vesicle preparations derived from rat liver have been useful to distinguish transport across the cell membrane from intracellular events. They may be selectively enriched in basolateral or apical plasma membrane, or in intracellular membranes[77]. The first identification of liver anion exchange activity was carried out in canalicular plasma membrane vesicles[78].

While isolated couplets of hepatocytes was particularly useful as primary secretory units to study canalicular secretion[79], the development in the last decade of the model of isolated bile duct units (IBDU), mainly from rat[16,80,81], but also from mouse[82] is making a great contribution to better study the regulation of ductal bile secretion[83]. Both models permit micropuncture for electrophysiological studies as well as video microscopic optical planimetry to determine bile secretion[16,81,84]. Isolated cholangiocytes[85-89] are also widely employed for transport studies. The preparation of different size subpopulations of cholangiocytes and IBDU isolated from specific portions of the rat intrahepatic biliary tree has made it possible to define the functional heterogeneity of cells lining specific sized intrahepatic ducts[2,90]. Furthermore, knockout and mutant animal models are being used to isolate cholangiocytes and bile duct units to carry out different *in vitro* studies[91-93]. Moreover, studies have been performed in isolated cholangiocytes from patients with cytic fibrosis (i.e. patients with mutations in the *CFTR* gene)[94,95]. Finally, a major advance is coming from the development of polarized primary cultures of intrahepatic cholangiocytes from both rat[96,97] and humans[98-101].

As the excretion of bicarbonate to bile involves a change in intracellular pH (pH_i) and a counterbalance through ion transporters in the responsible epithelial cells, several procedures and maneuvers have been developed to study pH_i regulation. The main strategy is based on the pH_i recovery towards its initial values after loading cells with acid or alkali. This type of experiment requires techniques for rapid and efficient monitoring of the pH_i. Although the most direct and accurate method seems to be the insertion of double-barreled microelectrodes sensitive to $[H^+]_i$ into the cell, this has important limitations; tested cells should have large dimensions and a special ability to manipulate the electrodes. Thus a valuable alternative is microfluorimetry, a procedure based on the sensitivity of intracellular fluorescent dyes to pH_i. The indicator most widely employed is the membrane-permeable compound 2', 7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM)[102-104]. Uncharged BCECF-AM rapidly enters the cell, where it is enzymatically cleaved into its charged, membrane impermeant, fluorescent form BCECF. When bound to H^+ ions, BCECF exhibits a shift in its emission fluorescence spectrum, and the ratio between the pH-sensitive emission at 495 nm wavelength, and the pH-insensitive (background) emission at the isosbestic point (440 nm) can be estimated. Once proper adjustments are set up (correcting for cellular autofluorescence, minimizing dye leakage, bleaching, or compartmentation, and preventing photodynamic damage to the cells), this methodology allows for continuous measurements of pH_i, with a very rapid response time. The technique can be applied to studies with a fluorimeter, flow cytometer, inverted epifluorescent microscope connected to a photon-counting photometer and a TV camera attachment plus a digital image-processing software[105]. An example of successful use of this methodology is the measurement of the Na^+-independent Cl^-/HCO_3^- anion exchange (AE) activity in both cell clusters and single cells. After inducing intracellular alkalinization by administration and further withdrawal of the cell-permeant weak acid propionate in Krebs-Ringer bicarbonate buffer (KRB), it is possible to estimate the anion exchange activity as the rate of spontaneous recovery of pH_i[106]. Rates of pH_i recovery can be measured as $\delta pH_i/\delta t$ from the tangent to the experimental plot; transmembrane acid fluxes (or equivalent transmembrane base fluxes, i.e. J_{OH}) are usually calculated as $\beta_{tot} \times \delta pH_i/\delta t$, β_{tot} being the total intracellular buffering power in the presence of CO_2/HCO_3^-, estimated as described[107,108].

The collection of additional important methodologies which enabe a continuous progress in bile secretion pathophysiology is still large. Thus, microcomputed tomography[109], scanning and transmission electron microscopy[89,110], immunoelectron microscopy[111], and dual labeled immunogold[112,113] are only a few among those techniques deserving a brief mention. Moreover, concerning molecular biological tools, the techniques of gene silencing through RNA-interference show great potential for clarifying the function of selective genes in bile duct cells. Currently, RNA-interference may be achieved with small interfering RNAs (siRNA) and through microRNAs (miRNA) and short/small hairpin RNA (shRNA)[114]. siRNAs had been used in normal rat IBDU to examine the role of fibrocystin in ciliary morphology and biliary cystogenesis[57] and that of aquaporin-1 (AQP1) in the transport of water by biliary epithelia[115]. Also siRNA experiments had been carried out in cholangiocarcinoma cells to identify factors involved, for example, in the growth of these cells[116] or their resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)[117]. Usual transfection protocols are not very efficient to internalize the siRNAs in cultured

cholangiocytes. Viral vectors that incorporate shRNA expression cassettes have thus been developed. Recently, a recombinant adenovirus with this design has been constructed and employed to efficiently infect normal rat cholangiocytes in culture and test the function of AE2/SLC4A2 anion exchanger in these cells[17].

MECHANISMS INVOLVED

Bile formation is regarded as an osmotic water flow in response to active solute transport. Bile salts are secreted in the canaliculi through a specific export pump referred to as BSEP/SPGP/ABCB11 (from bile salt export pump, sister of P-glycoprotein, and ATP-binding cassette, subfamily B, member 11, respectively)[118-120], allowing for the generation of the bile salt-dependent flow fraction. Remaining bile-salt independent flow fractions can be driven by supplementary solutes secreted at both the canaliculi (the so-called canalicular bile salt-independent flow), and the bile ducts (named as ductal bile salt-independent flow)[1,2,10,14,121]. Estimates for the magnitude of each bile flow fraction in humans are shown in Table 1.

Table 1 Estimated bile flow rates in humans[10]

Bile flow fraction	Flow rate
Canalicular bile acid-dependent flow	0.15-0.16 mL/min
Canalicular bile acid-independent flow	0.16-0.17 mL/min
Ductal secretion of bile flow	0.11 mL/min
Daily total bile flow	620 mL/d

Table 2 Estimations of canalicular bile-salt independent flow in different species

Species	Flow rate[122] (μL/min/kg)	Percentage of the canalicular bile flow
Humans	2	30%[128,129]
Monkey	7	
Dog	5	20%[9]
Rat	70	> 60%[1,131,132]
Rabbit	60	> 50%[133]

Canalicular bile-salt independent flow

In addition to bile acid secretion, the canalicular membranes of hepatocytes show active secretion of other organic and inorganic compounds, mainly glutathione[13] and bicarbonate[11,12,122], respectively. Glutathione can be secreted *via* the organic anion transporter MRP2/ABCC2[123], while the efflux of bicarbonate occurs through a DIDS-sensitive Na^+-independent Cl^-/HCO_3^- exchange in association with other ion transport systems (Figure 1)[78,124-127]. Both glutathione and bicarbonate seem to have an equivalent major input in canalicular bile flow generation, each driving up to 50% of the bile salt-independent fraction[11]. For this to be accomplished, resultant osmotic forces need to be associated with aquaporin-mediated transcellular movement of water molecules from plasma to the bile canaliculi[128]. In any event, the relative contribution of the canalicular bile salt-independent fraction to the whole canalicular bile flow may vary substantially between animal species (Table 2).

Canalicular bicarbonate excretion has been reported to be regulated by glucagon[134,135], a hormonal oligopeptide secreted by the pancreatic α-cells. This hormone is encoded by the gene *GCG*, which belongs to the same multigene family as secretin (*SCT*), vasoactive intestinal peptide (*VIP*), and gastric inhibitory peptide (*GIP*) genes, among others. Following its synthesis in the pancreas, glucagon may reach the liver and stimulate the hepatocytes *via* its interaction with the glucagon receptor (Figure 2). Interestingly, this receptor and the receptors for secretin, VIP, GIP and other small peptidic hormones, are included in a superfamily of receptors characterized by a 7-transmembrane domain structure and by their coupling to adenylate cyclase (ADCY) *via* GTP-binding proteins (G proteins). Glucagon-glucagon receptor interaction in hepatocytes leads to increased intracellular levels of cAMP, PKA activation, stimulation of canalicular Cl^-/HCO_3^- exchange activity[126,134,135], and enhanced AQP8-mediated water permeability at the canalicular membrane[128,136-138]. Current data strongly suggests that these choleretic effects of glucagon are microtubular-dependent and involve mobilization of intracellular vesicles[126,128,136,139,140]. These effects of glucagon in hepatocytes resemble those of secretin in cholangiocytes (Figure 2). After their interaction with their specific G-protein-coupled receptors, both hormones appear to use a similar cAMP-dependent PKA pathway to co-redistribute cell-type specific intracellular vesicles with flux proteins towards the apical plasma membrane of the target cell. But some of the flux proteins involved in bile formation may differ between hepatocytes and cholangiocytes. While AQP8 is the involved water chanel in hepatocytes[128,136,137,140], it changes to AQP1 in cholangiocytes[112,141,142]. Moreover, there are data suggesting that in hepatic cells in baseline situation, the water channel AQP8, the glutathione carrier MRP2/ABCC2 and the chloride bicarbonate exchanger AE2/SLC4A2[138,140] are present in pericanalicular vesicles that might migrate to the canalicular membrane upon glucagon stimulation[128]. These data, together with previous findings on AE2/SLC4A2 expression at the canaliculi and the pericanalicular area[143,144], point towards the canalicular bicarbonate excretion occurring *via* an AE2/SLC4A2-mediated Cl^-/HCO_3^- exchange[127]. Although a number of observations suggest that this excretion of bicarbonate in exchange for chloride functions in connection with an apical chloride channel that maintains favorable Cl^- gradients[78,124,127], the specific chloride channel(s) involved need yet to be identified (see Figures 1 and 2). While in cholangiocytes AE2/SLC4A2 colocalizes with CFTR[112], this particular cAMP-responsive Cl^- channel is not expressed in hepatocytes. Therefore, it might be expected that a Cl^- channel other than CFTR[145-148] be physically and/or functionally associated with AE2/SLC4A2 in hepatocytes. Moreover, previous findings have established an important link between bicarbonate excretion to bile and changes in pH_i. Canalicular Cl^-/HCO_3^- exchange is a major hepatic acid loading mechanism; at resting pH_i and in the absence of any stimulation it shows low activity, but it is rapidly activated by intracellular alkalosis, whereas cAMP stimulation leads to intracellular acidification

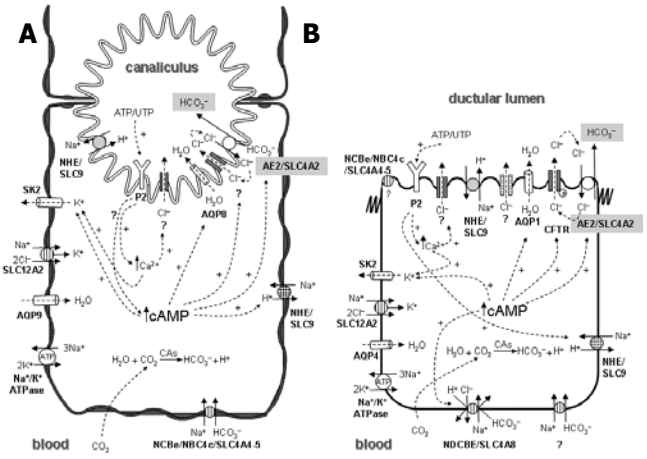


Figure 1 The major ion carriers implicated in pH_i regulation and secretion of bicarbonate to bile from liver cells. **A:** Hepatocytes; **B:** Cholangiocytes.

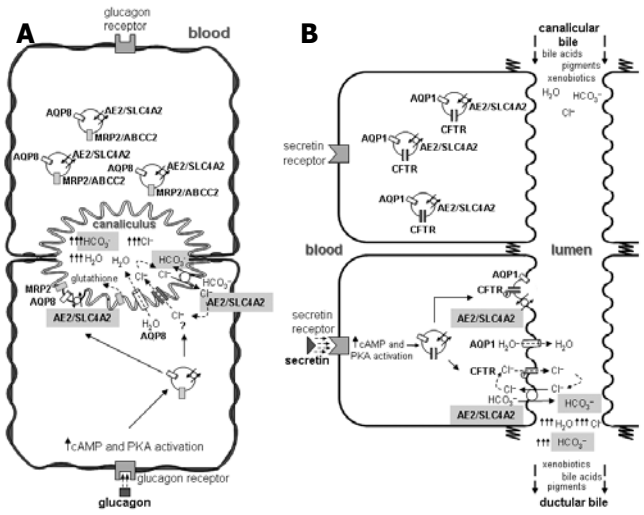


Figure 2 Hormonal stimulation of bicarbonate excretion from hepatocytes and cholangiocytes. **A:** In hepatocytes, glucagon may induce an exocytic trafficking of vesicles with the anion exchanger AE2/SLC4A2 and the water channel AQP8, which can results in canalicular bicarbonate-rich hydrocholeresis. MRP2/ABCC2, while being a carrier for organic anions like glutathione rather than an inorganic ion transporter, is hereby depicted because of its possible colocalization with the other two flux proteins; **B:** In cholangiocytes, secretin induces trafficking of vesicles with the chloride channel CFTR, the anion exchanger AE2/SLC4A2, and the water channel AQP1, and further exocytosis to the lumenal membrane of these cells, which results in ductal bicarbonate-rich hydrocholeresis.

which requires appropriate counterbalance[124,127]. Figure 1 summarizes several carriers putatively involved in these events in hepatocytes (as well as in cholangiocytes), including acid/base transporters and related proteins. A brief recall on bicarbonate loaders -and therefore acid extruders- highlights the role of electrogenic Na^+-HCO_3^- cotransporters (NBCe)[149-151] and the carbonic anhydrase/CA-CO_2 pathway[152,153]. Intracellular load of bicarbonate may increase upon hydration of intracellular CO_2 by carbonic anhydrase(s)[154] and subsequent H^+ efflux *via* Na^+/H^+ exchange (NHE) (Figure 1). The involved exchanger is mainly the basolateral NHE1/SLC9A1[155-157], but NHE4/SLC9A4[158] and the canalicular NHE3/SLC9A3[159] may also participate. The other relevant way of loading bicarbonate into hepatocytes, i.e. through electrogenic Na^+-HCO_3^- cotransport, functions upon membrane depolarization following intracellular acidification[160]. The specific proteins mediating this cotransport in hepatocytes remain to be defined. The only two members of the SLC4 family of bicarbonate transporters[161] known to be electrogenic are NBCe1/SLC4A4 and NBCe2/NBC4/SLC4A5[162]. NBCe1/SLC4A4 is widely expressed in most tissues, but its expression is negligible in the liver[163,164], while mRNA expression for NBCe2/NCB4/SLC4A5 is high in this tissue[165]. Of the known human NBCe2/NCB4/SLC4A5 variants, only the rat NBC4c ortholog can be detected in the rat liver by RT-PCR[164]. Moreover, NBC4c immunoreactivity had been observed at the basolateral membrane of rat hepatocytes[164]. Altogether these findings suggest that the basolateral NBC4c variant might be a relevant bicarbonate loader that enables hepatocytes for canalicular secretion of bicarbonate through the apical AE2/SLC4A2 anion exchanger.

Ductal bile

As previously mentioned, primary canalicular bile undergoes a process of fluidization and alkalinization along the biliary tract that is influenced by several factors including hormones (mainly secretin in most species[2,14-17]), innervation/neuropeptides[2,50,166,167], and biliary constituents[2,5,6,17,18]. This process results in net ductal secretion of a bicarbonate-rich watery fluid. The magnitude of the ductal contribution varies between species, representing 30% of basal bile flow in humans and 10% in rats[6]. Cholangiocytes are provided with specific transport systems that participate in bile modifications[2,168]. They are able to take up bile salts *via* an apical Na^+-dependent transporter (SLC10A2, formerly ASBT/ISBT and occasionaly ABAT)[45,169-172] and release them through a basolateral truncated isoform of the same carrier[173]. Indeed, transcellular transport through these carriers is specially important under cholestatic situations. In any case under normal conditions, biliary transport of bicarbonate appears as a relevant function of the bile duct epithelium. It is accomplished by specific acid/base carriers and related transporters that enable cholangiocytes to regulate their pH_i[105,174] (Figure 1).

Cholangiocyte acid extruders

The vectorial transport of HCO_3^- from cholangiocytes to duct lumen starts with the accumulation of HCO_3^- in the cells *via* mechanisms which vary between animal species. In rat cholangiocytes, for instance, HCO_3^- loading is mediated by transport systems similar to those in hepatocytes, i.e. by electrogenic Na^+-HCO_3^- cotransport activity[168,174-176] and the carbonic anhydrase/CA-CO_2 pathway coupled to subsequent carrier-mediated H^+ extrusion[153,154]. Carbonic anhydrases (CAs) catalyze the hydratation of carbon dioxide, $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$ (reviewed in ref. 153). Thus far, several CA isoenzymes have been identified which differ in organ distribution, subcellular location, and function (cf. Table I in ref. 153). Compared with other secretory organs, the mammalian liver contains relatively low levels of total CA activity. Thus, the cytoplasmic CA-II (CA2)

is mainly expressed in bile duct cells (but it may also be found in hepatocytes), and appears to be involved in the production of HCO_3^- for its further secretion to bile[153,177]. Also, the biliary epithelial cells express the membrane associated CA-IV and CA-IX, which on the basis of their location might be involved in acidification and concentration of the bile, even though the exact mechanisms have not been described[153].

Among electrogenic Na^+-HCO_3^- cotransporters[161,162] only the NBC4c variant of NBCe2/NBC4/SLC4A5 was found to be expressed in the rat liver, being immunolocalized to both hepatocytes and cholangiocytes[164]. Interestingly, NBC4c immunoreactivity was basolateral in hepatocytes but apical in cholangiocytes, suggesting a potential role for this cotransporter in the luminal fluid secretion and/or absorption[164]. In humans cholangiocytes, however, Na^+-HCO_3^- cotransport is not active in the physiological range of pH_i, being active only at very low pH_i, and bicarbonate influx occurs mainly through electroneutral Na^+-dependent Cl^-/HCO_3^- anion exchange[176,178]. Thus far, NDCBE/SLC4A8 is the only Na^+-dependent Cl^-/HCO_3^- exchanger cloned in humans[179]. Although Northern blot analysis could not detect its messenger RNA in the whole liver[179], this cannot rule out that NDCBE/SLC4A8 be expressed in cholangiocytes (which account for just 5% of the liver cell population).

In both rat and human cholangiocytes, H^+ extrusion takes place essentially through NHE activity (Figure 1) -while in pig cholangiocytes H^+ extrusion is mediated by a cAMP-activated H^+-ATPase[180]. Thus far, several members of the NHE/SLC9 family have been described in rat cholangiocytes. They include NHE1/SLC9A1, restricted to the basolateral membrane and highly sensitive to amiloride, and the amiloride-insensitive isoform NHE2/SLC9A2, which is likely to be active on the side facing the lumen[106]. Moreover, NHE3/SLC9A3 has been immunolocalized to the apical membrane of rat cholangiocytes, where it may play an important role in fluid absorption from the bile duct lumen[159]. Also NHE4/SLC9A4 -an isoform seemingly activated by hypertonicity and with K^+/H^+ exchange activity[181], was identified in whole liver extracts by Western blot[158]. Some findings in the stomach of *Nhe4/Slc9a4*$^{-/-}$ mice had led to speculations on a possible functional coupling of NHE4/SLC9A4 with the AE2/SLC4A2 anion exchanger in parietal cells[182]. In any case, the specific cellular expression and function of this NHE isoform in the liver remains to be determined.

Cholangiocytes acid loaders

As previously reported in hepatocytes, the main acid loader mechanism in bile duct cells is the apical Na^+-independent Cl^-/HCO_3^- exchange[17,106,174,176]. Such an AE activity might secrete HCO_3^- into the lumen which is exchanged for Cl^- influx. This exchange is electroneutral, being facilitated by the outside to inside transmembrane gradient of Cl^- at relatively high intracellular concentration of HCO_3^-, specially upon secretin stimulation[16,81,106,175,183] (Figure 2). Actually, several bicarbonate transporters have been described as exerting Na^+-independent Cl^-/HCO_3^- exchange activity. This is the case for the SLC4 anion exchangers (AE1/SLC4A1, AE2/SLC4A2 and AE3/SLC4A3)[161,184] as well as several members of the SLC26 gene family of multifunctional anion exchangers (DRA/CLD/SLC26A3, PDS/DFNB4/SLC26A4, and SLC26A6[185] and more controversially SL26A7[186,187]). But none of these carriers except AE2/SLC4A2 had been described to occur in the liver. Moreover, AE2/SLC4A2 was localized not only to the canaliculi but also to the lumenal membrane of bile duct cells[143]. Recent experiments of RNA intereference with recombinant adenovirus expressing shRNA have shown that AE2/SL4A2 is indeed the main effector of both basal and stimulated Na^+-independent Cl^-/HCO_3^- exchange in rat cholangiocytes[17].

Other ion-loaders/extruders

Besides acid/base transporters cholangiocytes possess other ion carriers like those for Cl^-, Na^+, and K^+, which greatly contribute to pH_i regulation and bicarbonate secretion (Figure 1). Thus, the cAMP-responsive Cl^- channel CFTR had been localized at the apical side, where it plays a role in biliary excretion of HCO_3^-[188,189]. Although HCO_3^- permeability through activated CFTR has been shown in several cell systems[190-194], its main contribution to biliary bicarbonate secretion appears to occur through a coordinated action with AE2/SL4A2[17,175,195]. In addition to CFTR, cholangiocytes possess a dense population of Ca^{2+}-activated Cl^- channels. These channels are responsive to interaction of the purinergic-2 (P2) receptors with nucleotides (mainly ATP or UTP)[196-199], and they appear to be related to the Ca^{2+}-calmodulin-dependent protein kinase II[188,200,201]. Resultant Ca^{2+}-stimulated Cl^- efflux might be up to 2-fold greater than that mediated by cAMP[202]. Additional high conductance anion channels which are insensitive to both Ca^{2+} and cAMP were identified in rat bile duct cells[203], but their specific role remains to be defined. The efflux of Cl^- from cholangiocytes to the ductular lumen is counterbalanced not only *via* apical Cl^-/HCO_3^- exchange but also through other Cl^- uptake systems, mainly the basolateral Na^+-K^+-$2Cl^-$ cotransporter NKCC1/SLC12A2[196,204]. In addition to maintain high intracellular Cl^- concentration facilitating apical Cl^- excretion, this forskolin-stimulable cotransporter has been reported to participate in cell volume homeostasis[205] and cell proliferation[204,206,207]. Because the NKCC1/SLC12A2-mediated influx of Cl^- occurs together with the entry of Na^+ and K^+, cholangiocytes possess other systems that sustain the gradients of these cations and the membrane potential difference. Thus a basolateral sodium pump (the Na^+/K^+-ATPase)[208,209], extrudes Na^+ and uptakes K^+ with a 3:2 stoichiometry[210]. Accumulation of K^+ within cholangiocytes is prevented by its exit through K^+ channels. Intracellular cAMP and/or Ca^{2+} concentration may activate basolateral K^+ conductance that hyperpolarizes the cell[211]. The small conductance K^+ channel SK2/KCNN2 has been identified in rat and human hepatocytes[212] and in rat and human biliary epithelia[202,212], the activity of which is stimulated by small increases in intracellular Ca^{2+} in an apamin-sensitive manner[213]. Although SK2/KCNN2 immunoreactivity has been localized in cholangiocytes at both apical and basolateral membranes, functional studies in polarized preparations have demonstrated a significantly greater basolateral Ca^{2+}-stimulated K^+ conductance[202].

Because the nonselective K^+ channel blocker Ba^{2+} is able to cause a greater degree of inhibition than apamin[202], apamin-insensitive channels not yet identified may also be involved in the conductance of K^+.

In a context of cAMP- and/or Ca^{2+}-stimulated Cl^- channels, cell hyperpolarization due to K^+ conductance facilitates the transcellular Cl^- movement into the lumen. Recycling of K^+ can be held up through the basolateral Na^+-K^+-$2Cl^-$ cotransport and the Na^+/K^+-ATPase. All these ionic fluxes across cholangiocyte membranes may ultimately lead to biliary excretion of bicarbonate *via* its exchange with lumenal chloride, which is facilitated by the outside to inside transmembrane gradient of chloride at relatively high intracellular concentration of bicarbonate[16,81,106,175,183]. The apical fluxes of anions result in increased osmotic forces in the bile duct lumen which in the presence of aquaporins contribute to the generation of ductal bile flow. This view has been strongly supported by the finding that AE2/SLC4A2 and CFTR both colocalize with AQP1 in cholangiocyte intracellular vesicles which co-redistribute to the apical cholangiocyte membrane upon both cAMP and secretin stimulations[112].

Water transporters or aquaporins

Aquaporins are water channels that mediate a bidirectional passive movement of water molecules across epithelial cells in response to osmotic gradients established by ions and solutes. Thus far, rodent cholangiocytes have been described to possess two types of aquaporins. Thus, in addition to the AQP1 locating in the intracellular vesicles which may traffic to the apical membrane upon agonist stimulation[112], there occurs another water channel at the basolateral membrane of cholangiocytes named AQP4, which is not sensitive to secretin[214,215] (Figures 1 and 2). However, there can be differences between species. For instance, immunohistochemical analysis in liver pig has shown the presence of AQP1 and AQP9 in cholangiocytes, while AQP3, AQP4, AQP7, and AQP8 were absent in these cells[216].

REGULATORY FACTORS

The bile duct epithelium is constantly regulated by the action of multiples factors that contribute to the formation of bile flow with an adequate final composition. Among these factors secretin is a relevant hormone peptide which may induce bicarbonate-rich hydrocholeresis in many animal species. Thus, secretin regulation of ductal bile flow and the concurrence of other factors are briefly summarized as follows:

Relevance of secretin stimulation

In 1902, Bayliss and Starling[19] described an active agent originating from the intestinal mucosa, which they referred to as secretin because of its capacity to stimulate pancreatic secretion in the dog. They also used the word "hormone" to designate this sort of compound that can be produced in an organ and carried through the circulation to exert its effect on another organ. Some years passed before the choleretic effect of secretin in the liver was reported in several animal species and humans[217-219]. Secretin was found to stimulate bile flow together with a decrease in the concentration of bile salts[217-219]. In the late 1960s the role of bile ducts in secretin-induced hydrocholeresis was postulated because of the observation that this secretin effect was associated with reciprocal changes in the biliary concentration of bicarbonate and chloride anions[35].

Meanwhile secretin was purified from most species, first in 1962 from pig[220,221] and then from humans, dog, goat, guinea pig, rabbit, rat, mouse and chicken. Secretin has only 27 amino acids, which allowed for its chemical synthesis as early as 1968[222]. There is close homology between mammalian secretins, but also between the regulatory peptides (more than 10) currently grouped in the secretin/glucagon/ VIP superfamily.

Secretin is produced in many organs but mainly in the mucosa of upper small intestine (duodenum and jejunum)[223,224]. Its release to blood occurs mainly in the postprandial period, being stimulated by gastric acid delivered into duodenal lumen[225], as well as by pancreatic and intestinal secretions[226,227]. Secretin release appears to be mediated by a luminal secretin-releasing peptide contained in these gastrointestinal juices[228-230]. In addition to its mentioned bilary and pancreatic effects, secretin may function as feedback inhibitor of gastrin (GAST) release and gastric acid secretion[231], and may also regulate gastric motility[232]. The physiological activities of secretin are subjected to hormone-hormone and neuro-hormonal interactions. Inhibition of gastric acid secretion by secretin is thus mediated by somatostatin (SST) and prostagladins[233,234], and secretin inhibition of gastric motility involves a vagal afferent pathway[235,236].

Secretin exerts its physiological actions *via* interaction with the N-terminal extracellular tail of its specific glycoprotein receptor SCTR[237-239]. Like the glucagon receptor and other members of the same receptor super-family, SCTR is coupled to adenylate cyclase/ADCY through an oligomeric GTP-binding protein. In the liver SCTR is exclusively expressed at the basolateral membrane of cholangiocytes[240,241]. As previously noted, the action of secretin in cholangiocytes runs parallel with the choleretic effect of glucagon in hepatocytes (Figure 2). Thus, both glucagon and secretin may stimulate the bile salt-independent bile flow, but each at a different level, i.e. canalicular for the former and ductular for the latter(cf. ref. 242).

Secretin-SCTR interaction in cholangiocytes results in increased intracellular levels of cAMP[15,16,243]. Further PKA activation[175,183] can induce microtubule-dependent co-redistribution of the intracellular vesicles with AE2-CFTR-AQP1 flux carriers to the apical membrane[112]. Additionally, the CFTR is phosphorylated and activated[244], thus resulting in Cl^- efflux to the ductular lumen. In consequence, Cl^- secreted by CFTR can be exchanged with HCO_3^- through AE2/SLC4A2. This mechanistic model has been consistently confirmed *in vitro* using rat IBDU[16,175] and cholangiocytes[174]. For *in vivo* studies, most experiments with rats have used some of the aforementioned models with bile duct proliferation[41,43,46,112,245,246], since normal rats appear to respond very poorly to secretin (likewise rabbit, but in contrast with guinea pig among rodent species)[15,41,46,47,247]. Indeed the expression of

SCTR is increased in BDL rat cholangiocytes[245,246] but the receptor is also expressed in normal rat cholangiocytes[240]. In the model of IPRL[248], intra-arterial secretin increased biliary concentration of bicarbonate, but had no effect on the net bile flow. Because the effect of secretin was blocked only by the CFTR inhibitor NPPB and not by the anion exchanger inhibitor DIDS (both administered intra-arterially), it was proposed that Cl^-/HCO_3^- exchange would have no role in the ductal secretion of bicarbonate in the normal rat[248]. However, recent experiments indicate that secretin does increase bile flow and biliary Cl^- and HCO_3^- excretions in the normal rat, but when they maintain the bile acid pool *via* continuous infusion with taurocholate[17]. Moreover, these effects of secretin were distinctively blocked by the inhibitors given by intrabiliary retrograde injection. While secretin effects were all blocked by intrabiliary NPPB, DIDS only inhibited secretin-induced increases in bile flow and bicarbonate excretion but not the increased chloride excretion[17]. These findings provide evidence for the role of biliary Cl^-/HCO_3^- exchange in secretin-induced bicarbonate-rich choleresis in the normal rat model.

The role of the bile acid pool

In line with former findings in earlier experiments with dogs[14,249], the aforementioned observation that secretin also has effects on the normal rat when infused continuously with taurocholate[17] confirms the notion that bile acids are relevant for secretin actions. In a previous study in normal rats secretin and taurocholate were tested and no secretin effects were observed[41]. But in that study, taurocholate infusion was interrupted before secretin administration[41]. It is already known that bile acids can enter into cholangiocytes through the carrier ASBT[45,169-172] and exert their effects on these cells (reviewed in refs. 2 and 250). For instance, it has been recently reported, activation of CFTR by ASBT-mediated bile salt absorption, which is seemingly independent from cAMP or cGMP signaling[251].

The bile salt-dependent canalicular flow is related to the osmotic activity of bile acids, but some bile acids such as ursodeoxycholic acid (UDCA), 23-nor-UDCA, and 23-nor-chenodeoxycholate[252-254], have a higher choleretic effect than can be accounted for by their secretion into bile (the so-called hypercholeretic effect). This hypercholeretic effect is associated with a marked stimulation of bicarbonate secretion into bile. A classic hypothesis referred to as "cholehepatic shunt pathway"[253] claimed that the hypercholeretic effect may involve intraductal protonation of unconjugated bile salts which results in the formation of bicarbonate anions derived from hydrated CO_2 (i.e. H_2CO_3 or H^+ plus HCO_3^-). Passive diffussion of uncharged bile acids through cholangiocytes to the periductular vessels and further uptake in hepatocytes could be followed by their canalicular resecretion as unconjugated bile salts (reviewed in ref. 250). After the identification of apical and basolateral bile acid carriers in cholangiocytes (ASBT and tASBT, respectively[45,169-173]), the cholehepatic shunt hypothesis has received a boost, being updated for the conjugated bile salts[250]. ASBT activity acutely increases upon secretin stimulation[172], which may accentuate the cholehepatic bile acid shunting in the postprandial period.

Neurovegetative liver innervation

The liver is directly regulated by the neurovegetative innervation. Indeed, the release of the neurotransmitter acetylcholine from the intrahepatic parasympatic terminals induces, *via* selectively interaction with M_3 Ach receptors on cholangiocytes, an increase in both secretin-stimulated cholangiocyte cAMP synthesis and Cl^-/HCO_3^- exchanger activity by Ca^{2+}-calcineurin-mediated PKC-independent modulation of adenylate cyclase/ADCY[255,256]. In fact, infusion of acetylcholine in the IPRL model was found to potentiate the effect of secretin on biliary HCO_3^- excretion[248]. Furthermore, interruption of parasympatic innervation in BDL rats by vagotomy has been reported to inhibit secretin-stimulated ductal secretion, as well as to decrease cholangiocyte intracellular cAMP levels[50].

The intrahepatic biliary epithelium also receives dopaminergic innervation[257,258], but in contrast to the cholinergic system, the dopaminergic system inhibits secretin-induced choleresis. Although both systems exert their functions through increased intracellular ions (1, 4, 5) P_3 and Ca^{2+}, the cholinergic system acts *via* calmodulin and calcineurin but without recruitment of PKC[256], whereas the D_2 dopaminergic system inhibits secretin-stimulated ductal secretion through increased expression and activation of PKC-γ[259].

Moreover, cholangiocyte secretion can also be regulated by the action of the adrenergic system[260,261]. The α_2-adrenergic receptor agonist UK-14304 has been reported to inhibit cholangiocarcinoma growth through time course-dependent modulation of Raf-1 and B-Raf activities[260], and the α_1-adrenergic agonist phenylephrine can potentiate secretin-stimulated ductal secretion through the amplification of the ADCY system *via* a Ca^{2+}- and PKC-dependent mechanism[261].

Hormones

In addition to secretin, there are gastrointestinal hormones and neuropeptides such as bombesin/gastrin releasing peptide (BN/GRP), VIP, endothelin-1 (ET1/EDN1), somatostatin/SST, and gastrin/GAST, which may also modulate the ductular bile salt-independent flow (reviewed in ref. 2). Some of these factors operate through a secretin-independent mechanism, while others influence the release of secretin or interact with the secretin signaling in cholangiocytes, depending very much on the animal species. For instance, the neuropeptide bombesin/BN/GRP can act either by increasing the secretin release in dogs[262,263], or inducing ductal secretion with activated Cl^-/HCO_3^- exchange *via* secretin-independent mechanisms in isolated rat cholangiocytes[2,264-266]. The effect of VIP on cholangiocytes depends also on the animal species[2,267-271]. While VIP appears to increase secretin-stimulated bile flow and bicarbonate excretion in humans[267,268], studies in rat IBDU show that VIP can stimulate basal fluid and bicarbonate secretion through a cAMP-independent pathway[2,269].

The cyclic tetradecapeptide somatostatin/SST is able to inhibit basal and secretin-stimulated bicarbonate-rich choleresis *via* its interaction with the SSTR2 receptor

subtype[2,272]. In rat, inhibition of secretin-stimulated ductal secretion by SST is associated with decreased expression of the secretin receptor SCTR in cholangiocytes and reduced secretin-stimulated cAMP levels[49,272]. In mice SST has also been shown to stimulate ductal fluid absorption, a process involving intracellular cGMP synthesis and inhibition of secretin-stimulated cAMP synthesis[273]. Moreover, in dogs, SST has been shown to diminish the acid-induced release of secretin from the duodenal mucosa[262]. Similarly SST, the gastrointestinal hormone gastrin/GAST may also modulate cholangiocyte secretion[2]. In BDL rats, GAST has been reported to inhibit secretin-stimulated ductal secretion by reducing both SCTR expression and secretin-induced cAMP levels[274].

Other peptide hormones like insulin and insulin-like growth factor 1 (IGF1) can also modulate the biliary epithelium. Insulin was reported to inhibit secretin-induced secretion in BDL rats through activation of PKC and inhibition of secretin-stimulated cAMP and PKA activity[275]. On the other hand, studies using a liver cell line (though from hepatoma rather than cholangiocytic type), showed that insulin may stimulate membrane turnover with increased exocytosis/endocytosis of vesicles containing ion channels[276]. In rats IGF1 was found to stimulate choleresis[277] as well as cholangiocyte proliferation[278]. IGF1 can be synthesized and released from cholangiocytes under the control of the growth hormone (GH)[278]. Biliary IGF1 may in turn interact with its receptor (IGF-R) located at the apical pole of cholangiocytes[278]. Expression of both IGF1-R and IGF1 is markedly enhanced in cholangiocytes following bile duct ligation[278].

Also steroidal hormones like corticosteroids and estrogens have effects on the biliary epithelium. Corticosteroids are choleretic and increase biliary bicarbonate excretion[277,279]. However, estrogen-induced cholestasis results in diminished biliary bicarbonate excretion[280]. Reduced bicarbonate excretion might be caused by a reflux of biliary bicarbonate *via* leaky tight junctions as it is not associated with impaired activity of the Cl^-/HCO_3^- exchanger[280].

Purinergic stimulation

Both hepatocytes and cholangiocytes are able to release ATP and UTP, which leads to increased biliary concentration of nucleotides and nucleosides (the latter being a result of nucleotide dephosphorylation by membrane-associated nucleotidases)[281-283]. Stimulation of the different subtypes of purinergic receptors (PR) at the lumenal membrane of cholangiocytes by either extracellular nucleosides (P1 family receptors) or extracellular nucleotides (P2 family receptors) may control cholangiocyte secretion and ion channel activities[281,283-286]. Most subtypes of purinergic receptors are G protein-coupled receptors except the P2X subtypes which are ligand-gated channels[281,283]. P2 activation stimulates cholangiocyte biliary efflux of K^+, HCO_3^-, and Cl^-, and reabsorption of Na^+[281,283,286]. Whereas Cl^- efflux seems to be mediated by a calcium-stimulated Cl^- channel[284], HCO_3^- is secreted from cholangiocytes *via* an AE2/SLC4A2 mediated Cl^-/HCO_3^- exchange[17,105,174,284,287]. Of notice, the $P2Y_{11}$ subtype receptor has been reported to mediate secretion *via* cAMP in pancreatic duct epithelial cells[288], which suggests that a similar mechanism may also occur in cholangiocytes (as well as in hepatocytes). Finally, P2 receptors may stimulate the basolateral NHE1/SLC9A1 activity in cholangiocytes[287].

Cytokines and related factors

Some cytokines such as IL5 and the combination of the proinflammatory cytokines IL6, IFN-γ, IL1, and TNF-α can inhibit secretin-induced choleresis[289,290]. Moreover, proinflammatory cytokines can impair the barrier function of biliary epithelia[290], and stimulate the biliary epithelium to generate NO, *via* induction of inducible nitric oxide synthase 2A (NOS2A/INOS). Resultant reactive nitrogen oxide species (RNOS) may cause ductular cholestasis through inhibition of both the soluble adenylate cyclase (SAC) and the cAMP-dependent HCO_3^- and Cl^- secretory mechanisms[195]. Such a pathogenetic sequence may contribute to ductal cholestasis in inflammatory cholangiopathies[195].

In conclussion, biliary secretion of bicarbonate is an important contributor to the generation of the bile-salt independent flow. Bicarbonate is secreted from both hepatocytes and cholangiocytes through parallel mechanisms which involve chloride efflux through activation of Cl^- channels, and further bicarbonate excretion *via* AE2/SLC4A2-mediated Cl^-/HCO_3^- exchange. Glucagon and secretin are two relevant hormones which act very similarly in hepatocytes and cholangiocytes, respectively. These hormones interact with their specific receptor, resulting in increased intracellular cAMP levels and activation of cAMP-dependent Cl^- and HCO_3^- secretory mechanisms. Both hepatocytes and cholangiocytes seem to have cAMP-responsive intracellular vesicles in which AE2/SLC4A2 may colocalize with cell specific Cl^- channels (CFTR in cholangiocytes and thus far undetermined in hepatocytes) and aquaporins (AQP1 in cholangiocytes and AQP8 in hepatocytes). cAMP-induced coordinated trafficking of these vecicles to either canalicular or cholangiocyte lumenal membranes and further exocytosis results in increased osmotic forces and passive movement of water with net bicarbonate-rich hydrocholeresis.

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Biliary wound healing, ductular reactions, and IL-6/gp130 signaling in the development of liver disease

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Abstract

Basic and translational wound healing research in the biliary tree lag significantly behind similar studies on the skin and gastrointestinal tract. This is at least partly attributable to lack of easy access to the biliary tract for study. But clinical relevance, more interest in biliary epithelial cell (BEC) pathophysiology, and widespread availability of BEC cultures are factors reversing this trend. In the extra-hepatic biliary tree, ineffectual wound healing, scarring and stricture development are pressing issues. In the smallest intra-hepatic bile ducts either impaired BEC proliferation or an exuberant response can contribute to liver disease. Chronic inflammation and persistent wound healing reactions in large and small bile ducts often lead to liver cancer. General concepts of wound healing as they apply to the biliary tract, importance of cellular processes dependent on IL-6/gp130/STAT3 signaling pathways, unanswered questions, and future directions are discussed.

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Key words: Biliary wound healing; Ductular reactions; IL-6/gp130 signaling

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INTRODUCTION

Wound healing in the biliary tract significantly contributes to the development of liver disease. For example, ineffectual wound healing, mural scarring, and stricture development in the large extra-hepatic bile ducts are responsible for diseases such as extra-hepatic biliary atresia and primary sclerosing cholangitis. These are leading indications for liver transplantation (http://www.optn.org). A similar problem occurs in 10%-15% of all liver allografts-the biliary sludge syndrome. Conversely, an exuberant wound healing response, or ductular reaction, contributes to the development of cirrhosis from a variety of causes[1-3].

BEC lining the extra-hepatic and large intra-hepatic bile ducts have a different embryologic origin and are distinct phenotypically from BEC lining small intrahepatic bile ducts. But considerations with respect to wound healing are similar to each other and to wound healing at other sites. Unique aspects of the biliary tract that have the potential to impact significantly on wound healing include: (1) anatomy and physiology; (2) exposure to high concentration of bile; and (3) triggering of reactions in the smallest intra-hepatic bile ducts by insults other than, or in addition to, direct injury and ulceration. In addition, chronic inflammation and persistent wound healing reactions in either the large or small bile ducts often precede the development of cancers.

In the skin and intestines, mechanisms of wound repair depend on the depth of injury. Superficial wounds or simple erosions are healed primarily by a two-step process: restitution and regeneration. Restitution begins immediately after creating a superficial wound in barrier epithelia. Cells near the edge of the defect lose close contacts with neighboring cells, undergo shape changes, spread, migrate, and then contract to close the hole. However, restitution is necessarily limited because remaining cells can cover only so much of the denuded surface area. In large wounds, regeneration or proliferation of the remaining epithelial cells is also needed. Eventually the epithelium is restored to a nearly original state[4,5], although surviving cells may carry a legacy of DNA damage and senescence-related changes[6]. Deeper wounds involve the epithelia and underlying stroma. Processes such as angiogenesis; activation, migration, and proliferation

of (myo-) fibroblasts and endothelial cells; formation of granulation tissue; and wound contraction are needed to close these larger/deeper defects. These more extensive wounds are also frequently inflamed and, in general, stromal involvement and inflammation greatly increase the risk of subsequent scarring[7-10].

Epithelial aspects of wound repair are often studied, *in vitro*, by producing linear "wound" tracks in confluent epithelial monolayers. The restitution phase is isolated by treating the cultures with chemical mito-inhibitors to prevent cell proliferation from contributing to wound closure. Distances migrated by the epithelial cells from the edge of the wound at predetermined time points measure the effectiveness of restitution. We developed a BEC model using a collagen-matrix substrate to prevent premature BEC senescence, which occurs routinely when BEC are plated on plastic or collagen-coated plates, from interfering with the assay[11].

"Front row cells", or those epithelial cells nearest the defect, experience dissolution of epithelial cell-cell contacts and changes in cell shape[5,12]. They can also acquire some mesenchymal characteristics and, under some circumstances, can undergo complete epithelial-mesenchymal transition (EMT)[13,14]. Changes in front row cells and EMT function to disaggregate epithelial units and reshape the epithelia for movement. Epithelia in transition lose polarity, adherens junctions, tight and gap junctions, desmosomes, and down-regulate cytokeratin intermediate filaments in order to rearrange their F-actin stress fibers and express filopodia and lamellopodia[5,12-14]. When wound closure is complete and proliferation has replenished the lost cells, re-establishment of inter-epithelial junctions restores the barrier. Coordinating these processes is critically important for barrier adaptation and wound healing[13,14].

POTENTIAL INFLUENCES OF BILIARY TREE PHYSIOLOGY AND ANATOMY ON WOUND HEALING

The biliary tree can be thought of as a delicate, relatively complex, self-contained organ that communicates with, and is enveloped by, the liver (Figure 1). It monitors, alters the composition of, and triages bile into the intestine. The tenuous only arterial blood supply can be damaged easily by diseases and by surgical procedures. A close relationship and cross-talk between BEC and periductal (myo-)fibroblasts exists throughout the entire biliary tree: damage to one population usually results in reactive changes in the other. For example, periductal (myo-) fibroblasts often undergo activation and proliferation in response to significant BEC growth, injury, and bile leakage from the small[15-21] or large bile ducts [22-24]. In extra-hepatic and large intra-hepatic bile ducts, this results in mural stricturing and luminal narrowing. In smaller intra-hepatic bile ducts, liver fibrosis and/or obliteration of the bile duct lumen can occur. A rich lymphatic network envelopes bile ducts that drains into regional hilar lymph nodes[25,26]. Numerous intramural peribiliary glands in the extra-hepatic bile ducts can become walled off after trauma and produce mucoceles. All of these potential

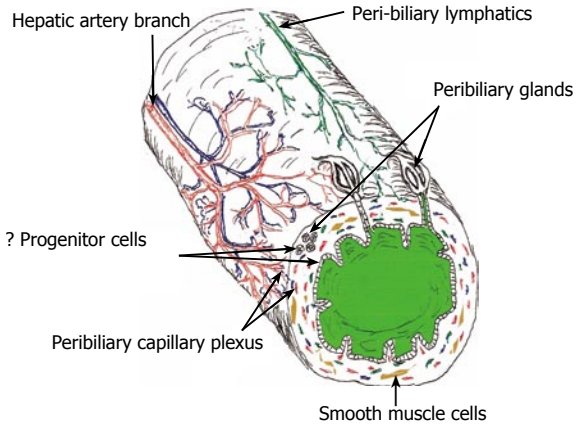


Figure 1 Diagram of the extra-hepatic and large intra-hepatic bile ducts highlighting some important anatomic and physiologic considerations that can potentially impact wound healing. Except for the peribiliary glands and some features of the BEC, small intra-hepatic bile ducts have a similar anatomy. All of the bile ducts, including the small intra-hepatic bile ducts are supplied only by the hepatic artery and the peribiliary vascular plexus, shown in red. All bile ducts also contain either smooth muscle cells and/or facultative myofibroblasts in their wall. Deep wounds to the biliary tree result in activation and/or transformation of myofibroblasts that greatly increase the risk of wound contraction, fibrosis, and stricture formation.

sources of problems contribute to the well-deserved moniker of the biliary tree as the "Achilles heel" of liver transplantation.

Bile contains bile salts that can induce[27-30] or protect BEC from apoptosis[31], cross-activate EGFR *via* TGFα ligand binding[32], induce COX-2 expression[33], or trigger BEC IL-6 and other cytokine production[34]. Bile also normally contains several growth factors (e.g. HGF), cytokines (IL-6), and other molecules[35]. Understanding the effect of various bile constituents on wound healing (esp. restitution) is critical because bile composition can be altered therapeutically (e.g. treating patients with ursodeoxycholic acid) [36,37].

BEC lining the smallest intra-hepatic bile ducts are derived from hepatoblasts and are thought to contain a population of liver stem cells that can differentiate into either hepatocytes or BEC[38-41]. Changes in the intra-hepatic environment other than, or in addition to, direct injury and ulceration can trigger wound repair reactions in these smallest ducts. These "ductular reactions" are recognized as BEC and surrounding myofibroblasts at the interface zone of diseased livers[3,42,43]. Ductular reactions can be provoked by: (1) local BEC injury and inflammation[44,45]; (2) increased intra-biliary tract pressure, and (3) the combination of: (a) a strong liver regenerative stimulus, such as partial hepatectomy or chronic necro-inflammatory liver disease and (b) hepatocyte mito-inhibition because of carcinogen exposure or chronic oxidative stress[1-3]. Insufficient BEC regeneration in the smallest ducts leads to liver diseases such as chronic "ductopenic" rejection and drug-induced ductopenia[44].

WOUND HEALING IN THE EXTRA-HEPATIC BILIARY TREE

Importance of arterial blood flow and wound depth

Study of the biliary sludge syndrome in liver allografts

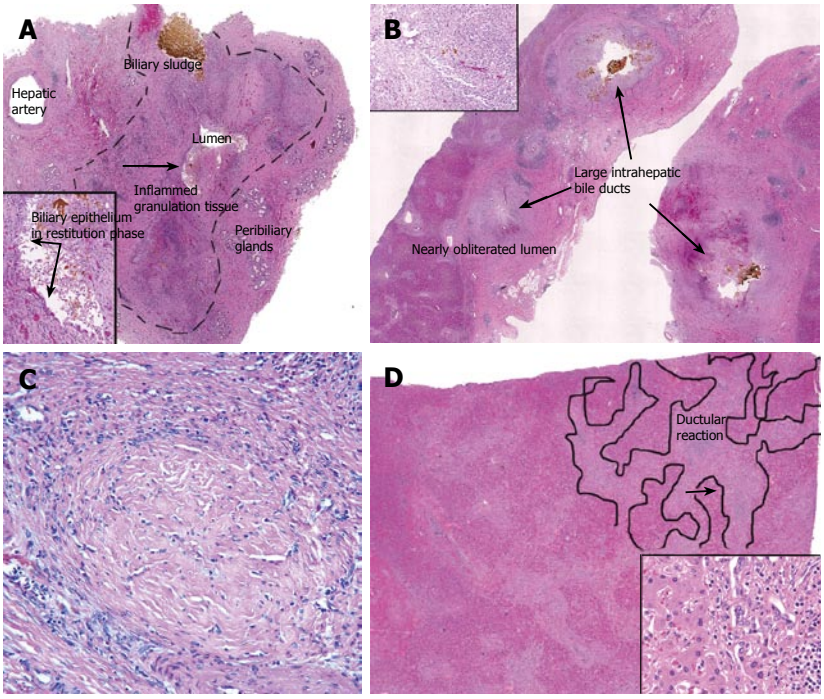


Figure 2 A: Cross-section of an extra-hepatic bile duct (outlined by dashed lines) from a liver allograft that failed because of the biliary sludge syndrome. Note the hepatic artery branch traveling along the outer wall, the peribiliary glands, mucosal ulceration, and that the lumen is nearly obliterated. Ulceration exposes the underlying stroma to bile, which results in inflammation, granulation tissue, and myofibroblast activation and proliferation in the underlying stroma. The inset shows at higher magnification the area near the arrow; note the bile sludge, BEC in restitution phase, and stromal inflammation and granulation tissue (arrows); **B**: Cross-section of several large intra-hepatic bile ducts from the same liver. Note that the same processes are occurring in these ducts. The duct lumen in the lower left is nearly obliterated by inflamed granulation tissue and myofibroblasts (inset); **C**: The final stages of "pathologic wound healing" in the intra-hepatic ducts can result in complete fibrous obliteration of the bile duct lumen by concentric rings of fibrous tissue, as shown here; **D**: Sections from the periphery of the same liver show a prominent ductular reaction consisting of BEC and periductal myofibroblasts (inset). This occurs because of increased pressure in the biliary tree distal to the site of luminal obliteration. Notice that the cholangioles and myofibroblasts form a wedge of tissue that arises from the portal tract and distorts the liver architecture (outlined on the right side of image).

has been particularly illustrative of pathophysiologic mechanisms involved in biliary wound healing. This relatively common and particularly frustrating complication affects about 10% of all liver allografts. There are many potential causes of ineffectual biliary wound healing including abnormal anatomy created by the operation; suboptimal arterial blood flow because of technical problems, anastomotic narrowing, thrombosis, or antibody mediated rejection; recurrent ascending cholangitis; recurrent primary sclerosing cholangitis; and ischemic-preservation injury[46]. Regardless of the cause, impediments to bile drainage results in progressive intrahepatic fibrosis, which in turn, increases morbidity and decreases organ half-life.

The extra-hepatic biliary tree is sustained only by the hepatic artery, which drains into three terminal classical capillary microvascular networks that supply: (1) the bile ducts (peribiliary plexus), (2) the connective tissue of the portal tracts, and (3) the hilar and perihilar structures[47]. The allograft biliary tree is especially vulnerable to arterial ischemia for the first several months after the operation. Preservation injury damages the microvasculature of the peribiliary plexus. The transplant operation can injure the arterial blood supply and it can also disrupt the normal collateral circulation typical of the arterial cascade arrangement supplying all gastrointestinal organs, including the liver[46]. Interference with arterial flow at any level can result in "ischemic cholangitis" - a succinct phrase used to describe the common association between poor arterial flow and biliary ischemia that manifests as persistent ulcers, inflammation, sludge, and strictures[48,49].

Cold ischemic-preservation injury depletes energy stores in microvascular endothelial cells and BEC. This results in activation of metalloproteinases, detachment of endothelium and BEC from the underlying matrix, and in the microvasculature, predisposition to thrombosis after reperfusion[50,51]. Reperfusion with blood after transplantation also delivers leukocytes that become activated by tissue damage. Activated leukocytes release effector molecules, which in turn, cause more tissue damage and further promote thrombogenesis[50,51]. Hydrophobic bile salts remaining in the biliary tree also further damage marginally viable BECs[50,52] already weakened by preservation injury. Damaged BEC are sloughed into the bile[53]. Although patient and allograft survival during the first several weeks after transplantation are dependent primarily on parenchymal function, long term allograft viability is determined primarily by biliary wound healing and adequate bile drainage[54,55]. Allografts that eventually fail show biliary sludge, mucosal ulcers, and inflamed granulation tissue and myofibroblast activation/proliferation in the wall of extra-hepatic and large intra-hepatic bile ducts[55] (Figure 2). Exposure of the underlying stroma to bile appears to serve as a nidus for crystallization of biliary sludge and a stimulus for inflammation and activation of myofibroblasts. This leads to wound contraction and fibrosis, and eventually, to strictures in large caliber ducts and to luminal obliteration of smaller caliber ducts (Figure 2C).

Two observations illustrate the critical and primary importance to wound healing of an adequate arterial blood supply. First, any insult that directly interferes with the arterial flow is usually associated with large bile duct ulcers and strictures. Second, perfusion of the hepatic artery and peribiliary plexus with low viscosity preservation solutions before transplantation dramatically decreases the incidence of biliary complications after transplantation in otherwise susceptible extended criteria donor livers with long cold ischemic times[56]. This maneuver is thought to flush thrombogenic material from the peribiliary plexus and facilitate reperfusion and oxygenation after transplantation. As with many clinical observations, confirmation of this mechanism is lacking. But it is reasonable to conclude that without sufficient arterial flow biliary wound healing is

unlikely to proceed normally. Once adequate arterial flow is ascertained, other factors that also significantly contribute to BEC wound repair can then be studied in greater detail.

Deep wounds of extra-hepatic bile ducts precipitate stromal involvement in wound healing. Granulation tissue and inflammation, local production of interferon-γ[57] and transforming growth factor beta (TGF-β)[58] at this site are of importance in wound contraction and scarring. Our laboratory has focused primarily on BEC aspects of wound repair with an emphasis on interleukin-6/gp130 signaling-dependent cellular processes. This signaling pathway is also critically important for wound healing in the gastrointestinal tract[59] and skin[60,61].

Cellular and molecular mechanisms of BEC repair involving IL-6/gp130 signaling

IL-6/gp130 is pleiotropic cytokine signaling system that has diverse effects in many different organ systems and cell types(reviewed in[62,63]). gp130 is one of the most promiscuous cytokine receptors[62], binding to many different ligands including, interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC)[62,63]. Ligand binding to gp130 generates two major signaling pathways: (1) Jak/STAT3 and (2) SHP2/ERK/MAPK and there is reciprocal negative feedback regulation between them[63,64]. Non-canonical STAT3 signaling pathways also exist, but are not well delineated[65].

In normal livers, IL-6 is produced at low levels by the BEC, perhaps stimulated by the bile salts[34], and secreted into the bile[66]. Active IL-6/gp130/STAT3 signaling can be detected in normal IL-$6^{+/+}$ but not in normal IL-$6^{-/-}$ mice livers, as evidenced by the detection of phospho-STAT3 by Western blotting and immunohistochemistry. Biliary tree pSTAT3 in normal liver localizes to occasional BEC lining large bile ducts, but more prevalent expression is seen in BEC lining the peribiliary glands[11].

Virtually any bile duct insult, such as obstruction[67-69], infection[69,70], or immunologic damage[66,71,72] triggers sharp increases in IL-6 mRNA and protein production by BEC and peribiliary hematolymphoid cells[3]. This, in essence, alerts BEC to environmental stimuli and leads to subsequent autocrine, paracrine, and juxtacrine gp130/STAT3 signaling in BEC at the sites of injury[11,67,68,73]. As in the gastrointestinal tract[59] and skin[60,61], an absence of IL-6 in IL-6-deficient (IL-$6^{-/-}$) mice leads to impaired wound healing[11] and biliary tree integrity[11,73,74]. For the last several years our laboratory has focused on cellular and molecular mechanisms that might contribute to impaired BEC wound healing and biliary barrier defects in the IL-$6^{-/-}$ mice and how the findings might apply to humans. Using a combination of knowledge gained from the gastrointestinal tract and skin, mRNA microarray expression analyses, and pathophysiologic studies, we first searched for genes that were: (1) expressed in BECs, (2) regulated by IL-6/gp130/STAT3 signaling, and (3) possibly involved in barrier function and/or repair. Two of the most interesting candidates, studied in greater detail, included intestinal trefoil family factors (TFF)[59,75-78] and small protein rich proteins (SPRR)[11,73]. The reader is referred to recent reviews of IL-6/gp130/ SHP2/ERK/MAPK signaling and other growth factors and cytokines involved in the regenerative phase of BEC wound healing[3,67,68,79-81].

Trefoil family factor (TFF) proteins are comprised of one or more trefoil motifs, which consist of 6 cysteine residues. TFF proteins increase mucous viscosity and thereby contribute to optimal protection of the intestinal mucosa from injury[77,82-84]. By enhancing intestinal epithelial spreading and migration, TFF proteins also stimulate the restitution phase of wound healing[76,85]. Each of the three known TFF proteins is differentially regulated in the gastrointestinal tract[59,75]: TFF1 and TFF2 are expressed primarily in the stomach[86,87]. TFF3 predominates in the small and large intestines[78] and in the biliary tree[11] of mouse and human livers[88-91]. IL-6/gp130/STAT3 signaling is crucial for BEC TFF3 expression. In normal liver, pSTAT3 and TFF3 mRNA and protein expression are significantly higher in IL-$6^{+/+}$ than in IL-$6^{-/-}$ mice livers. Constitutive expression of TFF3 and pSTAT3 localizes to mucin secreting BEC lining large intrahepatic and extrahepatic bile ducts and peribiliary glands[11]. Medium and small-sized intra-hepatic bile ducts are generally negative for mucin secreting BEC, pSTAT3, and TFF3 expression[11,91,92].

IL-$6^{+/+}$ BEC consistently show higher levels of TFF3 mRNA and protein expression and significantly better migration and wound healing than IL-$6^{-/-}$ BEC[11], *in vitro*. Defective migration in the IL-$6^{-/-}$ BEC can be partially, but significantly, reversed by treatment with recombinant TFF peptides[11]. *In vivo*, biliary TFF3 is dynamically regulated by various factors after bile duct ligation. Included are the reciprocal negative regulation known to exist between the STAT3 and MAPK signaling pathways[59], and other cytokines and growth factors, such as HGF and TGF-β, which can down-regulated BEC TFF3 expression[11]. However, a chronic deficiency of pSTAT3 signaling during bile duct injury, as seen in IL-$6^{-/-}$ mice after bile duct ligation, leads to a chronic deficiency of biliary TFF3 expression and impaired biliary barrier function[11]. In humans, p-STAT3 and TFF3 are newly co-expressed in BEC involved in florid duct lesions in primary biliary cirrhosis and at other sites of BEC injury, but not in similarly-sized normal bile ducts from the same livers[11,89,92]. This likely constitutes a primitive or innate mucosal defense system that guards against injury and stimulates repair.

Our BEC TFF3 studies are consistent with studies focused on the colon and carried out in mice harboring mutations that selectively block all gp130-mediated STAT activity ($gp130^{\Delta STAT}$), but preserve gp130-mediated MAPK signaling. These mice show decreased colonic TFF3 expression, increased sensitivity to sodium dextran sulfate-induced colitis, and impaired mucosal wound healing[59,75]. Thus, it is reasonable to conclude that IL-6/gp130/STAT3 signaling contributes significantly to normal BEC cytoprotective mechanisms and to migration during wound healing, at least in part, by stimulating BEC TFF3 expression[11].

Small proline-rich proteins (SPRR) are encoded by a tandemly arranged four-member gene family

contained within a 170-kilobase region of the epidermal differentiation complex (EDC). The EDC is a cluster of more than 50 genes located on chromosome 1q21[93-95] whose products are involved in terminal differentiation of the human epidermis. Included are formation of the cornified envelope that is an effective barrier against the external environment [93,96]. The four SPRR gene families, SPRR1-4, are distinguished on the basis of the number of amino acids in the repeats of the protein's central domain and the consensus of that sequence[95]. There are two Sprr1 genes and one copy each of Sprr3 and Sprr4 genes[95]. SPRR2 genes are the most diversified family: there are seven in humans and eleven in mice[95]. In the skin and other squamous epithelia, SPRR genes are usually regulated coordinately as part of the EDC (i.e. high expression of most EDC genes, as in papillomas, or very low expression of most genes, as in newborn skin). SPRR genes encode for a series of highly homologous proteins that function primarily as critical cross-linkers. They form bridges among other EDC proteins, intermediate filaments, and cornified envelope constituents[94,95,97], such as desmoplakin, loricrin, and tricohyalin, through the catalytic action of transglutaminases[98] .

The diverse SPRR2 genes are also non-coordinately expressed, or expressed preferentially, without similar upregulation of other EDC family members[73,97]. This occurs most commonly in non-keratinizing epithelia in curious situations that cannot be explained by squamous differentiation or formation of a cornified envelope. Examples include greater than 100-fold increases of SPRR2A in the intestine after small bowel resection[99] or after introduction of commensal bacteria into germ-free mice[100,101] or after intentional infection with intestinal parasites[102]. In uterine epithelium SPRR2A mRNA and protein expression is, at least in part, regulated by estrogen[103]. Therefore, it is highly and non-coordinately upregulated during certain stages of the oestrous cycle[104,105] and is especially high at the blastocyst implantation site[103]. SPRR2A mRNA and protein are also expressed in bronchial and intestinal epithelium during allergic reactions[106]. Barrier remodeling, as a response to stress[94,105], inflammation, and/or growth, is a common condition of these diverse circumstances. Potential molecular and cellular processes affected by non-coordinate SPRR2A expression are currently under investigation in our laboratory.

In the liver, we have shown that SPRR2A mRNA and protein are not expressed in normal mouse liver, but are non-coordinately upregulated only in BEC after the stress of bile duct ligation[73]. Expression after bile duct ligation is not related to squamous metaplasia and shows strong dependence on IL-6/gp130/STAT3 signaling. In BEC lining the large bile ducts, SPRR2 protein localizes subjacent to the apical plasma membrane. SPRR2 expression is more diffusely distributed throughout the cytoplasm of cholangioles participating in ductular reactions and in large duct BEC engaged in the restitution phase of mucosal wound healing. Deficient BEC *SPRR2A* expression in IL-$6^{-/-}$ mice after bile duct ligation is associated with impaired barrier function[73]. IL-6 replacement therapy restores *SPRR2A* expression to levels seen in wild type controls and reverses the barrier defect in IL-$6^{-/-}$ mice. In a series of ongoing investigations, preliminary data suggest that BEC SPRR2A expression is associated with BEC restitution.

WOUND HEALING IN SMALL INTRA-HEPATIC BILE DUCTULES-DUCTULAR REACTIONS

Wound healing responses can be triggered in BEC lining the smallest intrahepatic bile ducts by environmental changes other than, or in addition to, direct injury and ulceration. This often occurs in chronic necro-inflammatory liver disease regardless of the underlying cause. For several years we were puzzled by the observation that ductular reactions represent a survival advantage for BEC and myofibroblasts over hepatocytes, yet hepatocytes and BEC share the same responses to many cytokines and growth factors that are upregulated in chronic liver disease (e.g. HGF, EGF, IL-6, TGFβ, *etc*.[81,107]). Why then do BEC and myofibroblasts survive preferentially under these circumstances? To answer this question it is helpful to view chronic necro-inflammatory liver disease as a "Darwinian" selection pressure applied to the liver. A survival advantage for BEC can occur because of a relative increase in the rate of proliferation, a relative decrease in the rate of death, transformation of hepatocytes into BEC, or various combinations of the above. Regardless of the mechanism, the end result is a relative decrease in volume percentage of hepatocytes and a relative increase in biliary epithelial cells and myofibroblasts-a pattern typical of evolving cirrhosis[1,74]. When combined with sufficient time[1,2], even a small deficit of hepatocyte survival is enough to evoke a ductular reaction that distorts the hepatic architecture.

Using an established mouse model of decompensated biliary cirrhosis[74] and p21-deficient mice, we tested the hypothesis that hepatocyte mito-inhibition combined with the regenerative stimulus of bile duct ligation would accentuate the ductular reaction and accelerate architectural distortion. Results showed that after long-term (12-wk) ligation mice prone to decompensation show significantly more oxidative stress and hepatocyte nuclear p21 expression, a cyclin dependent kinase inhibitor and important mediator of hepatocyte mito-inhibition[1]. As expected, mice prone to decompensation also showed less hepatocyte proliferation, an exaggerated ductular reaction, and accelerated architectural distortion compared with compensation-prone controls[1]. We next subjected p21 deficient mice to bile duct ligation for 12 wk with the expectation that p21 deficient mice would be better able than wild-type controls to compensate for long-term BDL because of significantly greater hepatocyte proliferation. Indeed, results of these experiments showed that p21-deficient mice showed a larger liver mass because of more hepatocyte proliferation, a less florid ductular reaction, and less architectural distortion than wild type controls[1].

We next wanted to determine whether this concept was applicable to other, non-cholestatic or non-biliary, liver diseases. To accomplish this task, we first showed that hepatocyte nuclear p21 expression in humans awaiting liver replacement directly correlated with pathological disease stage and model of end-stage liver disease scoring[1]. We

also engaged in a collaborative study with Clouston *et al* who had previously shown that HCV-related liver disease progresses more rapidly when there is co-existent hepatic steatosis[108]. Liver biopsies from 115 patients with HCV scored for steatosis, inflammation, and fibrosis showed a strong correlation between (a) a ductular reaction and portal fibrosis and (b) steatosis and impaired hepatocyte replication[2]. Steatosis correlated with the ductular reaction and greater hepatic progenitor cell proliferation, but was not an obligate feature. The highly significant correlation between the ductular reaction area and fibrosis stage remained even after multivariate analysis[2]. Impaired hepatocyte replication, as determined by p21 expression, was independently associated with hepatic progenitor cell expansion, increased body mass index, and lobular inflammation.

The observation that ductular reactions often appear when hepatocyte mito-inhibition is combined with a liver regenerative stimulus is not new. It was made originally years ago while treating experimental animals with genotoxic carcinogens and then subjecting them to partial hepatectomy (reviewed in[3,109,110]). These maneuvers stimulate oval cell, or liver epithelial progenitor cell[111], expansion/proliferation at the interface zone-a ductular reaction. However, in carcinogenesis experiments, the ductular reaction eventually resolves without fibrosis.

Our studies show that this concept is applicable to ductular reactions associated with chronic necro-inflammatory liver diseases and the development of fibrosis/cirrhosis[1] (Figure 3). Hepatocytes are more susceptible to injury and mito-inhibition during chronic necro-inflammatory liver disease because they: (1) produce and secrete bile and are the major site of bile stasis; (2) are more complex metabolically and able preferentially to store lipids and metals, such as iron and copper, which are generators and/or catalysts for free oxygen radical formation; and, (3) support HBV and HCV replication, and (4) maybe most importantly, contain many more mitochondria than BEC and mitochondria are the major site of superoxide production[112]. Diverse disorders such as cholestasis[1], HCV replication[113,114], steatosis[2,115], copper deposition[116], and alcohol[117] preferentially stress or injure hepatocytes and this causes nuclear expression of the cyclin-dependent kinase inhibitor, p21[1,2], which in turn, inhibits hepatocyte proliferation.

When small intrahepatic bile ductules are destroyed (ductopenia), due to drugs or chronic allograft rejection, classical cirrhosis usually does not develop[45,109,118,119]. Despite ongoing immunologic liver injury and fibrosis, regenerative nodularity and the associated complications of portal hypertension rarely occur. BEC survival and proliferation in response to injury in these small ductules is related to a combination of immunologic injury, environmental influences, and importantly, arterial blood flow[120], as in the large bile ducts. Perhaps the arterial disease also inhibits regenerative nodule formation (Figure 4).

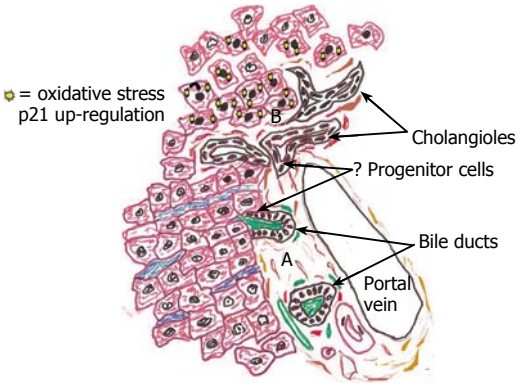


Figure 3 This diagram illustrates the peripheral aspects of the biliary tree, including the portal tract and periportal hepatic parenchyma in normal (A: bottom of figure) and in diseased livers (B: top of figure). A: Rare liver progenitor, or stem cells, are thought to reside within the Canals of Herring, or the portion of the bile ductules that connects BECs to hepatocytes. The same close relationship between BEC, the arterial supply, and periductal myofibroblasts seen in the large ducts continues to the biliary tree periphery, as shown here. B: During chronic necro-inflammatory liver diseases a variety of insults, such as cholestasis[1], HCV replication[114,115], steatosis[2,116] copper deposition[117], and alcohol[118] can cause oxidative stress, preferentially in hepatocytes. The stress upregulates hepatocyte nuclear p21 expression (illustrated by solid nuclei at top of diagram), which in turn, inhibits hepatocytes proliferation and accentuates ductular reactions[1,2]. A close relationship between BEC and periductal myofibroblasts in the smallest bile ductules usually results in co-activation of these populations recognized as ductular reactions, which often precede the development of cirrhosis.

PROGENITOR CELL ACTIVATION, PERSISTENT WOUND REPAIR AND CANCER

Persistent ductular reactions in human chronic necro-inflammatory diseases can activate progenitor cell populations, as in the experimental animal carcinogenesis models, discussed above (Reviewed in refs 38, 39, 41). Several groups, including ours, have shown that oval cell expansion in mice is dependent significantly on IL-6/gp130/STAT3 signaling[121,122]. This raises the possibility that ductular reactions accelerate the development of cirrhosis and potentially increase the risk of hepatocellular carcinoma. Since oval cells eventually differentiate into hepatocytes[111], exposure to carcinogens or genotoxic damage from oxidative stress imprint genetic mutations in putative liver stem cells. These cells then divide, differentiate, and spread initiated cells more widely throughout the entire hepatocyte population. Liver cancers occur when initiated hepatocytes are subjected to tumor promoters that generally cause hepatocyte proliferation. Chronic liver disease is an excellent cancer-promoting environment.

In the skin, STAT3 signaling enables epidermal stem cells to the escape apoptosis induced by exposure to cutaneous carcinogens[123]. Initiated stem cells survive, divide, differentiate, and subsequently give rise to skin cancers in a promoting environment[123]. Similar processes might occur in hepatocellular and cholangiocarcinomas. Interleukin-6/gp130/STAT3 signaling might provide important survival signals for initiated liver epithelial progenitor cells that later give rise to liver cancers in the context of chronic necro-inflammatory disease. In evolutionary biology[124] this process is referred as "antagonistic pleiotropy" - a short term survival benefit at the expense of long-term increased risk of cancer.

IL-6/gp130/STAT3 signaling is indeed increased in a very wide variety of neoplasms, including hepatocellular carcinomas, melanomas, leukemias and myelomas, and

lung, breast cancer, kidney, prostate, pancreatic, colon, gastric, cervical, ovarian, and head and neck cancers[125,126]. This signaling pathway participates and/or regulates many pathways important in oncogenesis including cell-cycle progression, apoptosis, tumor angiogenesis, tumor-cell invasion and metastasis, and tumor-cell evasion of the immune system (reviewed in[125,126]).

Whether this concept is applicable to cancers arising in extrahepatic and large intrahepatic bile ducts is uncertain because the mechanisms of BEC renewal at these sites have not been studied in any great detail. In the larger bile ducts there are two potential sources of new BEC: (1) proliferation of mature BEC; and (2) proliferation and/or maturation of progenitor cell populations. These potential sources are not mutually exclusive and only one study in the literature even indirectly addresses this topic. Koike *et al*[127] used pulse and continuous DNA labeling studies to show, in rats, that a proliferative zone, consistent with a BEC progenitor cell population, localized to peribiliary glands (called "crypts" in their study). Long-term follow-up of the animals showed that labeled BEC migrated gradually to the surface and were shed into the lumen with a transit time of about 30-40 d[127]. They concluded the arrangement and pattern of BEC renewal in the extra-hepatic bile ducts was similar to the intestine and colon, but kinetics of BEC turnover was slower[127]. More definitive work is needed in this area.

UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

The following are a few examples of the many unanswered questions in the study of biliary wound healing that have stimulated research in our laboratory. For example, are there progenitor cell populations within the peribiliary glands or crypts of extra-hepatic and large intra-hepatic bile ducts? If progenitor cells exist in the extra-hepatic biliary tree, where exactly do they reside, and how are they recognized? Are they activated during wound repair, and do they contribute to the development of bile duct cancers?

EMT contributes significantly to wound healing and to kidney fibrogenesis. Is EMT an important process in BEC wound healing and hepatic fibrogenesis? Can, and do, BEC transform into myo-(fibroblasts)? BEC appear to migrate and can acquire mesenchymal characteristics during the ductular reaction and hepatic fibrogenesis. They transform from polarized cuboidal epithelial cells into a spindle or dendritic-shaped vimentin-positive cells after bile duct ligation and in PBC[128,129]. In embryonic liver, ductal plate BEC "invade" or migrate into the portal tract connective tissue to form mature intrahepatic bile ducts. During migration BEC express vimentin[130] and down-regulate membranous E-cadherin[131] expression. Once mature bile ducts are formed BEC revert totally to an epithelial phenotype. Preliminary studies from our laboratory suggest that IL-6/gp130/STAT3 signaling triggers BEC changes under some circumstances that can induce BEC EMT. The extent to which EMT contributes to BEC wound healing, however, requires further study.

Several studies show that IL-6 pre-treatment can prevent liver parenchymal ischemic-preservation injury[132-136]. We have shown similar results in intestinal allografts: IL-6 pretreatment limits epithelial damage and promotes repair[137]. Would pretreatment of donor livers with IL-6, particularly in the aortic flush, have beneficial effects on preservation injury of the peribiliary plexus and promote wound healing in the biliary tree? We would expect IL-6/gp130 signaling to help lessen the incidence of biliary strictures because it upregulates anti-apoptotic molecules in the microvascular endothelium[133,136], preserves epithelial integrity[11,137], and stimulates BEC restitution[11,73] and regeneration[79] after injury.

Several other strategies might be used to prevent biliary strictures[138,139], such as reducing eosinophil and mast cell accumulation with Tranilast[140,141] and Captopril[142-144], reducing activation and transformation of myofibroblasts with anti-oxidants such as alpha tocopherol (vitamin E) and peroxisome proliferator-activated receptors-γ (PPAR-γ) ligands, such as the thiazolidinedione family of drugs[145-147].

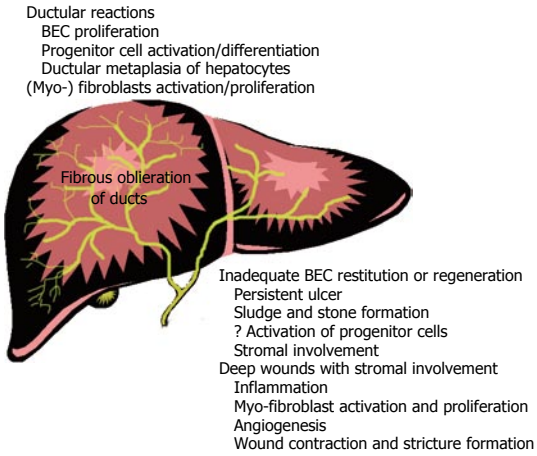


Figure 4 Summary of the most common "wound repair" reactions that contribute to the development of chronic liver disease and liver cancers. Although all of the processes described in the text can occur at any site in the biliary tree, certain types of responses are more common at certain sites. For example, in the extra-hepatic and large intra-hepatic bile ducts (lower right), inadequate or ineffectual epithelial restitution or proliferation results in persistent ulcers and stone and sludge formation. This, in turn, leads to possible activation of progenitor cells and stromal involvement, which can also be triggered by "deep wounding" of the large ducts. Stromal involvement is usually accompanied by inflammation, angiogenesis, and activation and proliferation of myofibroblasts. All of these processes contribute to wound contraction and stricture formation, which is one of the most common and clinically significant problems in the large ducts.

In the small bile ducts, ductular reactions are the most commonly encountered "wound healing" response. Ductular reactions can occur because of BEC proliferation, progenitor cell activation and differentiation, and ductular metaplasia of hepatocytes. They also usually trigger myofibroblast activation and proliferation and together these cell populations form a tissue wedge at the interface zone that progressively distorts the liver architecture.

In medium-sized bile ducts, fibrous obliteration is probably the most common serious wound healing response. This occurs because the response to injury of the periductal myofibroblasts overwhelms the BEC response and the duct lumen is obliterated.

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Heterogeneity of the intrahepatic biliary epithelium

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Abstract

The objectives of this review are to outline the recent findings related to the morphological heterogeneity of the biliary epithelium and the heterogeneous pathophysiological responses of different sized bile ducts to liver gastrointestinal hormones and peptides and liver injury/toxins with changes in apoptotic, proliferative and secretory activities. The knowledge of biliary function is rapidly increasing because of the recognition that biliary epithelial cells (cholangiocytes) are the targets of human cholangiopathies, which are characterized by proliferation/damage of bile ducts within a small range of sizes. The unique anatomy, morphology, innervation and vascularization of the biliary epithelium are consistent with function of cholangiocytes within different regions of the biliary tree. The *in vivo* models [e.g., bile duct ligation (BDL), partial hepatectomy, feeding of bile acids, carbon tetrachloride (CCl_4) or α-naphthylisothiocyanate (ANIT)] and the *in vivo* experimental tools [e.g., freshly isolated small and large cholangiocytes or intrahepatic bile duct units (IBDU) and primary cultures of small and large murine cholangiocytes] have allowed us to demonstrate the morphological and functional heterogeneity of the intrahepatic biliary epithelium. These models demonstrated the differential secretory activities and the heterogeneous apoptotic and proliferative responses of different sized ducts. Similar to animal models of cholangiocyte proliferation/injury restricted to specific sized ducts, in human liver diseases bile duct damage predominates specific sized bile ducts. Future studies related to the functional heterogeneity of the intrahepatic biliary epithelium may disclose new pathophysiological treatments for patients with cholangiopathies.

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Key words: cAMP; Gastrointestinal hormones; Growth factors; Mitosis; Nerves

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ANATOMICAL AND MORPHOLOGICAL CHARACTERISTICS OF THE BILIARY EPITHELIUM

Two kinds of epithelial cells, hepatocytes and cholangiocytes, are present in the liver[1-3]. While hepatocytes initially secrete bile into the bile canaliculus[4], cholangiocytes modify bile of canalicular origin by a series of coordinated spontaneous and hormone/peptide regulated secretion/reabsorption of water and electrolytes before it reaches the small intestine[3,5-7]. For more information on the mechanisms of bile formation we refer to recent reviews[4,5]. The human biliary system is divided into extrahepatic bile ducts and intrahepatic bile

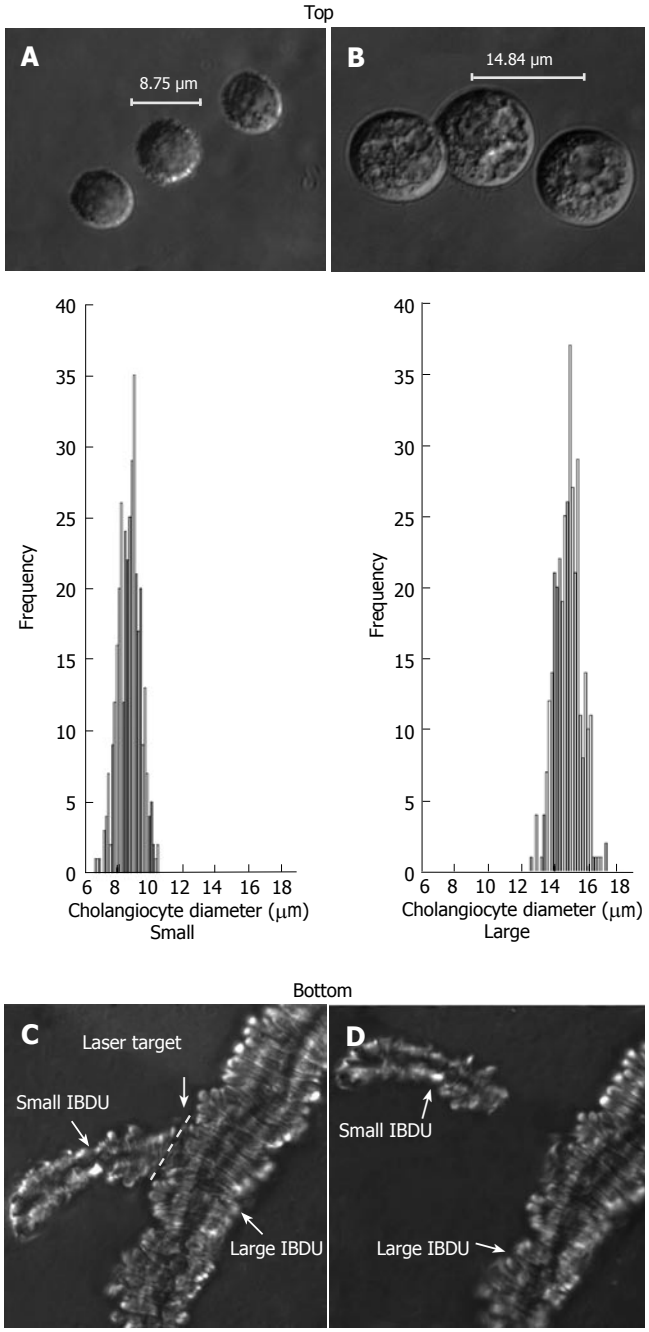


Figure 1 [**Top**] Isolation of small (**A**), approximately 8 μm diameter] and large (**B**), approximately 14 μm diameter] cholangiocytes from small and large ducts, respectively, from normal rats. Small and large cholangiocytes were purified by counterflow elutriation followed by immunoaffinity purification. Original magn., × 625. Reproduced with permission from Ref[12]. [**Bottom**] Isolation of small (**C**) and large (**D**) IBDU from normal rat liver. Small (< 15 μm in diameter) and large (> 15 μm in diameter) IBDU were pruned off from large ducts by a nitrogen pulsed dye laser and subsequently separated (**D**) by picking up IBDU with a micromanipulator micropipet. Original magnification × 2000. Reproduced with permission from Ref 13.

ducts, the latter further sub-divided into large and small bile ducts[2,3,8]. The intrahepatic bile ducts represent that part of the biliary tree proximal to the confluence of the hepatic ducts[9] extending from the canals of Hering to the large extrahepatic ducts[2,3,8]. In human liver, a study by Ludwig classified the intrahepatic bile duct system upon duct diameter[8], small bile ductules (< 15 μm), interlobular ducts (15-100 μm), septal ducts (100-300 μm), area ducts

Table 1 Terminology and relationship between human and rat intrahepatic bile ducts

Terminology for human bile ducts (diameter in μm)	Terminology for rat bile ducts (diameter in μm)
(Large bile ducts)	
Hepatic ducts (> 800)	
Segmental ducts (400-800)	
Area ducts (300-400)	
(Small bile ducts)	
Septal bile ducts (100-300)	
Interlobular bile ducts (15-100)	Large bile ducts (> 15)
Bile ductules (cholangioles) (< 15)	Small bile ducts (< 15)

These data have been obtained from studies[8,12,13] aimed to define the morphological characteristics of the biliary epithelium of rats, and humans. Reproduced with permission from Ref 2.

(300-400 μm), segmental ducts (400-800 μm) and hepatic ducts (> 800 μm)[8] (Table 1). Small ductules are lined by 4-5 cholangiocytes, have a basement membrane, tight junctions between cells and microvilli projecting into the bile duct lumen[10,11]. Cholangiocytes are progressively larger and more columnar in shape in larger bile ducts (lined by 10-12 cholangiocytes)[10,11].

In rats, morphological studies in liver sections and small and large intrahepatic bile duct units (IBDU) have shown[2,12-14] that the intrahepatic biliary tree is divided into: (1) small ducts (< 15 μm in external diameter) lined by small cholangiocytes (approximately 8 μm in diameter)[12,13]; and (2) and large ducts (> 15 μm in diameter) lined by large cholangiocytes (approximately 15 μm in diameter)[12,13] (Figure 1, Table 1). Specifically, we have shown[12] that the rat intrahepatic biliary epithelium is formed by ducts of different sizes (5 to 200 μm in external diameter) and cholangiocytes of different cell areas (3 to 80 μm^2). Furthermore, a direct relationship exists between cholangiocyte area and external duct diameter, a finding that demonstrates that small ducts are lined by small cholangiocytes, whereas larger ducts are lined by larger cholangiocytes[12-14]. The fact that small and large ducts are lined by small and large cholangiocytes, respectively, is important since it allows for the assignment of the secretory, apoptotic and proliferative functions (achieved in isolated small and large cholangiocytes) within the different portions of the intrahepatic biliary epithelium. Recently, Masyuk *et al*[15] have reconstructed the intrahepatic biliary epithelium that resembles a tree, with the common and hepatic ducts corresponding to the trunk, the intrahepatic bile ducts corresponding to the large branches and the small ducts corresponding to the smallest tree limbs of a tree.

Studies by Phillips *et al*[16] have shown that no major ultrastructural differences exist among cholangiocytes lining small and large bile ducts. However, in support of the concept that the intrahepatic biliary epithelium is morphologically heterogeneous, electron microscopic studies by Benedetti *et al*[14] in rat liver sections and IBDU have demonstrated that large bile ducts are lined by 8-15 cholangiocytes and small ducts by 4-5 cholangiocytes. The studies also showed that small and large cholangiocytes

have a multilobulated nucleus, numerous vesicles at the subapical region, tight junctions, high density of microvilli and lysosomes and a few mitochondria[14]. Other studies have shown the presence of microvilli and cilia in the apical plasma membrane of cholangiocytes[17,18], cilia that play an important role in the regulation of cholangiocyte functions[19,20]. While large cholangiocytes are columnar in shape, small cholangiocytes have a cuboidal shape[14]. Abundant Golgi apparatus was observed between the apical pole and the nucleus[14]. Rough endoplasmic reticulum was inconspicuous in the smallest ducts and increased only slightly in the largest[14]. While large cholangiocytes display a small nucleus and conspicuous cytoplasm, small cholangiocytes possess a high nucleus/cytoplasm ratio[14]. Cholangiocytes have distinct apical and basolateral membranes[14,17,18].Coated pits have also been observed on the apical and basolateral membranes of cholangiocytes, a finding suggesting receptor-mediated endocytosis at both domains of cholangiocytes[21]. Functional tight junctions are located between adjacent cholangiocytes in proximity to the apical domain[17].

INNERVATION

There is growing information regarding the role of the nervous system in the regulation of the pathophysiology of the biliary epithelium[3,22-27]. In the liver, adrenergic and cholinergic nerves are located around the hepatic artery, portal vein, and the biliary epithelium[28,29]. The intrahepatic arteries, veins, bile ducts and hepatocytes are also innervated[28,29]. In the autonomic nervous system, there are a number of regulatory peptides including neuropeptide tyrosine (NPY)[30,31], calcitonin gene related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide (VIP) (mostly associated with parasympathetic fibers), enkephalin and bombesin[31-35]. NPY-positive nerves are present in extrahepatic bile ducts[36] and have been suggested to regulate bile flow by autocrine/paracrine mechanisms[37]. We have shown that NPY inhibits cholangiocarcinoma growth by interaction with a G-protein coupled receptor by Ca^{2+}-dependent modulation of Src/ERK1/2 phosphorylation[38]. Nerve fibers containing CGRP and substance P are present around blood vessels and bile duct radicles within portal tracts[39,40]. VIP-positive nerve fibers are located in the walls of hepatic arteries, portal veins and bile ducts[41].

VASCULARIZATION

The intrahepatic and extrahepatic bile ducts are nourished by a complex network of minute vessels [*i.e.*, peribiliary vascular plexus (PBP)], which originate from branches of the hepatic artery and flow principally into the hepatic sinusoids, either directly (lobular branch) or by portal vein branches (prelobular branches)[42,43]. Since the blood flows in the opposite direction (from the large towards the small ducts) to bile flow, the PBP presents a countercurrent stream of biliary reabsorbed substances to hepatocytes[44,45]. We have previously shown that the function of the intrahepatic biliary tree is linked to its vascular supply sustained by the PBP[44]. Changes in intrahepatic bile duct mass are associated with changes of the PBP architecture[44]. Following BDL, the PBP undergoes hyperplasia, thus supporting the increased nutritional and functional demands from the proliferating bile ducts[44]. In support of this concept, studies[46] have shown that following chronic feeding of ANIT (which induces increases in both cholangiocyte proliferation/apoptosis)[47], the hepatic artery and portal vein undergo marked proliferation, presumably to support the increased nutritional and functional demands of the proliferated bile ducts[44,46]. However, the proliferation of the PBP occurs only after the hyperplasia of bile ducts[44]. Recent studies have shown that small and large rat bile ducts have a different vascular supply[44]. The PBP is primarily present around large bile ducts and less visible around small bile ducts[44], a finding that may partly explain why large but not small cholangiocytes proliferate following BDL in rats[48] and why small and large ducts differentially proliferate or are damaged in other experimental models of cholangiocyte proliferation/loss including chronic feeding of certain bile acids (e.g., taurocholate and taurolithocholate)[49], ANIT[47] or acute gavage administration of CCl_4[50,51] (Figure 2) or partial hepatectomy[52].

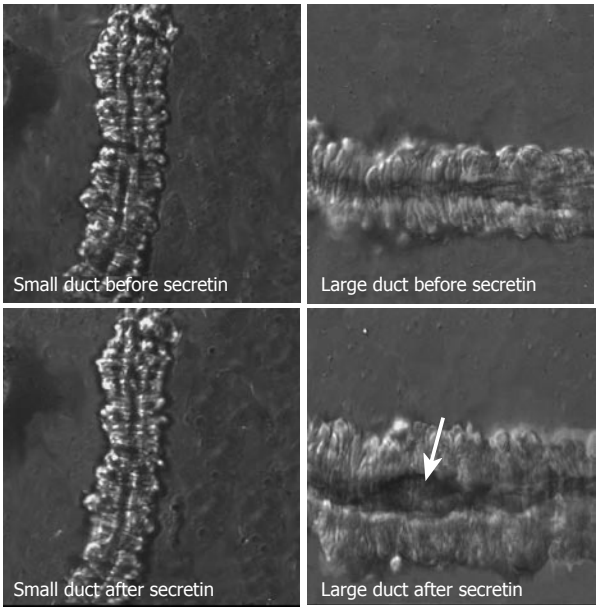


Figure 2 Measurement of H_3 histone gene expression in small and large cholangiocytes from 1-wk BDL rats and 1-wk BDL rats treated with CCl_4 or mineral oil. H_3 histone gene expression in large cholangiocytes decreased on d 2 before returning to control values on d 7 after CCl_4 treatment. H_3 histone gene expression (which was absent in small cholangiocytes from BDL rats) was expressed by small cholangiocytes on d 1 and 2 before returning to control undetectable values on d 7 after CCl_4 treatment. Administration of mineral oil to 1-wk BDL rats did not alter H_3 histone gene expression in large cholangiocytes. The message for H_3 histone gene was absent in small cholangiocytes from oil-treated rats. Comparability of RNA used was assessed by hybridization for GAPDH (housekeeping gene). Autoradiograms were quantified by densitometry. Densitometric values are means of 2 experiments. Reproduced with permission from Ref 50.

GENERAL BACKGROUND ON CHOLANGIOCYTE FUNCTIONS

The major function of cholangiocytes is to modify bile of canalicular origin[4] (by basal and hormone/peptide regulated secretion and reabsorption of

water and electrolytes) before reaching the small intestine[3,5,6]. Ductal secretion is coordinately modulated by gastrointestinal hormones (e.g., secretin, gastrin, insulin, somatostatin, bombesin and VIP)[3,5-7,12,53-58], gastrointestinal peptides (i.e., endothelin-1, ET-1)[59], enzymes (e.g., alkaline phosphatase)[60], bile acids (e.g., taurocholate, taurolithocholate, taurohyodeoxycholate, tauroursodeoxycholate, ursodeoxycholate and taurourso deoxycholate)[49,61-64] and cholinergic[23,26], adrenergic[65,66], serotoninergic[67] and dopaminergic[25] receptor agonists. Cholangiocytes, which have a low DNA turnover under normal physiological conditions[48,52,68], proliferate or are damaged in response to liver injury/toxins[2,3,6,26,47,48,50,52,68-72]. In rat liver, secretin is of particular importance since secretin receptors are only expressed by cholangiocytes[53], and its expression is upregulated under pathological conditions associated with enhanced cholangiocyte growth (e.g., after BDL)[48,71,72] and downregulated with cholangiocyte damage/loss (e.g., following acute CCl_4 administration)[50,51]. Thus, the secretin receptor is an important pathophysiological tool that allows us to evaluate the secretory, proliferative and apoptotic heterogeneity of the intrahepatic biliary epithelium in response to agonists and liver toxins/injury[2,3,12,13,47-51,54,61,71,73]. Interaction of secretin with its receptor is associated with increased intracellular cAMP levels[12,13,25,26,48,50-52,54,59,72]. Enhanced cAMP levels leads to phosphorylation of PKA[74], which induces opening of the cystic fibrosis transmembrane regulator (CFTR) channel[54] leading to the activation of the Cl^-/HCO_3^- exchanger[12,23,52] resulting in biliary bicarbonate secretion[6,52].

EXPERIMENTAL MODELS

A number of *in vivo* models (e.g., BDL, acute administration of CCl_4, partial hepatectomy, chronic feeding of ANIT or bile salts)[47-52] demonstrated that the intrahepatic biliary epithelium is functionally heterogeneous, with specific sized bile ducts (i.e., small and large) differentially responding to liver injury/toxins with changes in proliferative, apoptotic and secretory activities[2,3,12,47-52,54,62,71-73]. A number of *in vitro* experimental models (i.e., small and large cholangiocytes and IBDU and small and large immortalized normal murine cholangiocytes) (Figure 1)[12,13,47,48,50,51,75] have allowed us to suggest that the intrahepatic biliary epithelium is morphologically and functionally heterogeneous[2, 3,12,47-52,54,62,71-73]. The very first approach that was employed and that significantly contributed to lay down the basis of this field of research was the purification of small and large cholangiocytes from rat liver by counterflow elutriation[12,54,76]. Coupling such a technique to immunoaffinity separation[12,18,54], it was possible to isolate two distinct subpopulations of small (approximately 8 μm in diameter, obtained at the centripetal flow rate of 25 ml/min) and large (approximately 14 μm in diameter, collected at the flow rate of 55 mL/min) cholangiocytes (Figure 1)[12,54]. The two subpopulations of small and large cholangiocytes are further purified by immunoaffinity separation[18] using an antibody against an unidentified antigen (expressed by all intrahepatic cholangiocytes)[18] and characterized morphologically (by computerized image analysis) (Figure 1)[12,54], phenotypically (expression of γ-glutamyltransferase and cytokeratin-19 genes)[12,54] and functionally (by measurement of gene expression of secretin receptor, CFTR and Cl^-/HCO_3^- exchanger and basal and secretin-stimulated cAMP levels, Cl^- efflux and Cl^-/HCO_3^- exchanger activity)[12,54].

In addition, we have developed a technique for isolating small (diameter smaller than 15 μm) and large (diameter greater than 15 μm) IBDU from small and large bile ducts, respectively (Figure 1)[13]. This important tool allowed us to directly evaluate the differential secretory responses of different portions of the biliary epithelium to selected gastrointestinal hormones/peptides[13,25,65,77]. As shown in Figure 1, the small duct was pruned off from the large duct by a brief exposure of a laser focused on the junction between large and small ducts (arrow) leading to separation of small from large ducts[13]. Small and large IBDU were characterized by morphometric analysis, gene expression for secretin receptor, CFTR and Cl^-/HCO_3^- exchanger, secretin-induced cAMP levels, and secretion by change in luminal size in response to agonists including secretin, insulin, the α1-adrenergic receptor agonist, the α2-adrenergic receptor agonist, UK14,304 and the D2 dopaminergic receptor agonist, quinelorane[13,25,65,66,77].

Most recently. we have immortalized, from normal mice (BALB/c), small and large cholangiocytes by the introduction of the SV40 large T antigen gene, that allowed, after cloning, to establish small and large cholangiocyte cell lines[75]. The characteristics of the two subpopulations were evaluated by electron microscopy (EM) and measurement of trans-epithelial electrical resistance (TER), and secretin-stimulated cAMP levels[75]. EM, TER and differential cAMP response to secretin are consistent with the concept that small and large immortalized cholangiocytes originate from small and large ducts, respectively[75]. Microarray successfully displayed characteristic differential cDNA expression between small and large cholangiocytes[75]. Using the above described methods individually or in tandem, has allowed us to clearly demonstrate heterogeneity of the intrahepatic biliary epithelium and to dissect the differential physiological responses of these distinct subpopulations of cholangiocytes to endogenous stimuli.

HETEROGENEOUS EXPRESSION OF PROTEINS

The heterogeneous expression of some enzymes/proteins and membrane transporters/receptors in small and large ducts from mice, rats and humans is summarized in Table 2. In human liver, large septal bile ducts mainly express the sialylated $Lewis^a$ blood group antigen[78]. In normal and diseased human livers, hepatic, segmental, area, and septal bile ducts, and peribiliary glands express pancreatic enzymes such as pancreatic lipase, pancreatic α-amylase, and trypsin[79,80]. By microarray of RNA from small and large immortalized murine cholangiocytes, we have demonstrated the heterogeneous expression of approximately 80 proteins between small and large cholangiocytes[75]. The pathophysiological relevance of the

Table 2 Expression and function of proteins and surface transporters in small and large ducts from rats and human

Markers	Small ducts	Large ducts	Function	References
γ-glutamyl transpeptidase	Not expressed	Interlobular large rat bile ducts	Glutathione metabolism	[81]
Alkaline phosphatase	Not expressed	Interlobular large rat bile ducts	Inhibition of secretin choleresis	[81]
Leucine amino peptidase	Not expressed	Interlobular large rat bile ducts	Undefined	[81]
Cytochrome P4502E1	Not expressed	Expressed by large rat and human ducts	Dehalogenation of CCl_4	[50,81,104,105]
Lipase, α-amylase and trypsin	Human septal ducts	Large human ducts, and peribiliary glands	Biliary tree development	[79,80]
Bcl-2	Human small ductules	Not expressed	Anti-apoptotic protein	[109,143]
Secretin receptor	Not expressed	Expressed by large rat ducts	Stimulation of bicarbonate secretion	[12,13,48,54]
CFTR	Human but not rodent small ducts	Expressed by large rat ducts	Regulation of Cl^--secretion	[54]
Cl^-/HCO_3^- exchanger	Not expressed	Expressed by large rat and human ducts	Regulation of ductal bicarbonate secretion	[12,13,82]
Somatostatin receptor	Not expressed	Expressed by large rat ducts	Inhibition of secretin choleresis	[48]
D2 dopamine receptors	Unknown	Expressed by large rat ducts	Inhibition of secretin choleresis	[25]
α-1 adrenergic receptors	Expressed by small rat ducts	Expressed by large rat ducts	Stimulation of secretin choleresis	[65]
Endothelin receptors	Expressed by small rat ducts	Expressed by large rat ducts	Inhibition of secretin choleresis	[59]
Na^+-dependent ABAT	Not expressed	Expressed by large rat ducts	Regulation of ductal secretion	[88]

Heterogeneous expression of proteins and membrane transporters that may play a role in the modulation of the heterogeneous properties of the intrahepatic biliary tree of rats and human. Modified with permission from Ref 73.

differential expression of these messages remains to be addressed.

Secretory activity

Recent studies have demonstrated that large bile ducts are the major anatomical sites of cAMP-dependent ductal secretion by activation of cAMP/PKA/CFTR/Cl^-/HCO_3^- exchanger (Figure 3)[3,12,13,48,54]. Specifically, studies in isolated small and large cholangiocytes and IBDU from normal and BDL rats have shown that large (but not small) cholangiocytes express the messages for secretin receptor, CFTR and Cl^-/HCO_3^- exchanger and respond to secretin with increases in cAMP levels, Cl^- efflux and Cl^-/HCO_3^- exchanger activity and IBDU lumen expansion (Figure 3)[12,13,48,54]. In rat liver, large ducts express alkaline phosphatase and γ-glutamyltranspeptidase[81]. The expression of alkaline phosphatase in large ducts is consistent with our previous studies[81] showing that alkaline phosphatase inhibits secretin-stimulated choleresis by blockage of CFTR activity, which is expressed only in large ducts (Figure 3)[54]. Furthermore, large cholangiocytes (which is the only cholangiocyte subpopulation expressing the somatostatin receptor, $SSTR_2$)[48] are the major anatomical sites of somatostatin inhibition of secretin-stimulated ductal secretion (Figure 3)[48,55]. The inhibitory effects of somatostatin on secretin-stimulated secretion in large cholangiocytes are associated with reduced cAMP levels, Cl^- efflux and Cl^-/HCO_3^- exchanger activity[48,55,54]. The counter-regulatory effect of somatostatin on the choleretic effect of secretin is important in modulating ductal secretion in pathological conditions associated with cholangiocyte proliferation/loss[3]. Parallel with the findings observed in rat bile ducts[3,12,13,48, 4], in human liver secretin-stimulated duct secretory activity is heterogeneous, since only large bile interlobular ducts express the Cl^-/HCO_3^- exchanger[82].

We have demonstrated the presence of insulin and CCK-B/gastrin receptors in large cholangiocytes from normal and BDL rats and have shown that these two hormones inhibit secretin-stimulated ductal secretion of BDL rats by IP_3/Ca^{2+}/PKCα-dependent decrease of cAMP levels[7,72,77]. Similarly, we found that ET_A and ET_B receptors are expressed by large cholangiocytes and that ET-1 inhibits secretin-stimulated cAMP levels and ductal bile secretion of BDL rats by interaction with ET_A but not ET_B receptors[59]. Furthermore, recent data have shown that: (1) the D2 dopaminergic receptors are expressed by large BDL cholangiocytes; and (2) the D2 dopaminergic receptor agonist, quinelorane, inhibits secretin-stimulated ductal secretion by activation of the Ca^{2+}-dependent PKCγ[25]. The α2-adrenergic receptor agonist, UK14, 304, inhibits secretin-stimulated cAMP-dependent Cl^- efflux and Cl^-/HCO_3^- in large cholangiocytes and secretin-stimulated lumen expansion in large IBDU of BDL rats[66]. The α1-adrenergic receptor agonist, phenylephrine, stimulates cAMP levels and secretin-stimulated secretion of large BDL cholangiocytes by IP_3/Ca^{2+}-dependent activation of PKCα and PKCβ II[65]. We have recently demonstrated[26] that acetylcholine, by interacting with M3 receptor subtypes, potentiates secretin-stimulated cAMP levels and Cl^-/HCO_3^- exchanger activity in IBDU and purified cholangiocytes by a Ca^{2+}-calcineurin mediated but PKC independent modulation of adenylyl cyclase.

Following hepatocyte secretion[83], bile acids are reabsorbed by the biliary epithelium[84], then they return via the PBP to the hepatocytes for secretion into bile (cholehepatic shunting)[85]. As a mechanism for bile acids entry into cholangiocytes, the apical Na^+-dependent bile transporter, ASBT (structurally identical to the ileal bile acid transporter) is expressed on the apical membranes of large cholangiocytes[86]. Consistent with functional activity for ASBT in cholangiocytes, studies have shown Na^+-dependent and saturable uptake of taurocholate in normal

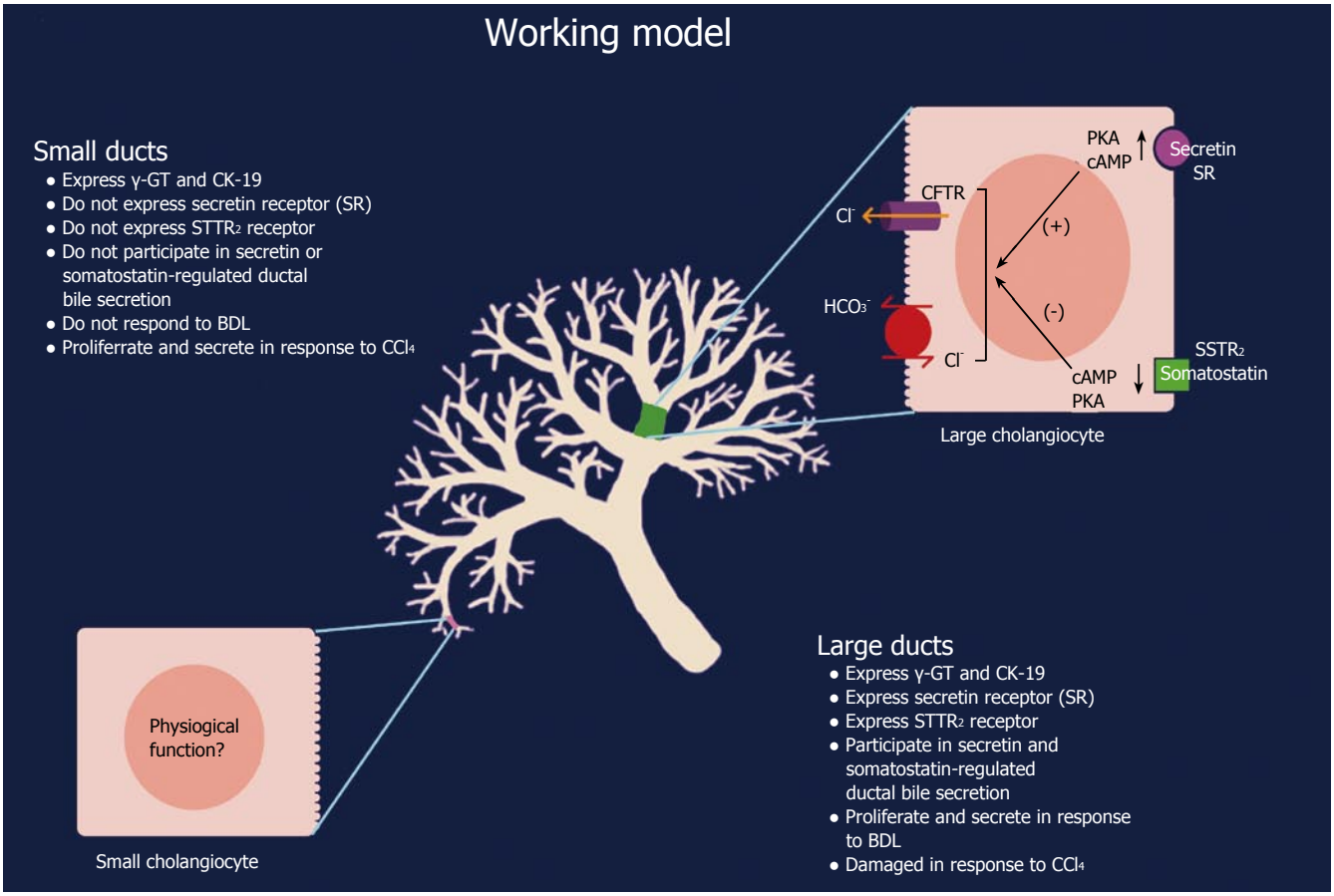


Figure 3 Working model for the heterogeneity of the intrahepatic biliary epithelium. The model proposes that: (1) bile ducts are morphologically heterogeneous with small ducts lined by small cholangiocytes and large ducts lined by large cholangiocytes; (2) small and large ducts similarly express both γ-GT and cytokeratin-19; and (3) large (but not small) ducts express the secretin and somatostatin receptor, CFTR and Cl^-/HCO_3^- and respond physiologically to these two hormones. The model also shows that following BDL, only large cholangiocytes proliferate and that a single dose of CCl_4 induces damage and loss of large duct function, whereas small cholangiocytes (resistant to CCl_4) *de novo* proliferate and secrete to compensate for the loss of large duct function. Reproduced with permission from Ref. 73.

cholangiocyte cultures[87] and large cholangiocytes[88]. These data suggests that after taurocholate and taurolithocholate enter into large cholangiocytes by ABAT, they stimulate secretin-stimulated ductal bile flow in these cholangiocyte subpopulations[88,89]. Other studies have shown that both taurocholate and taurolithocholate increase secretin-stimulated cAMP levels in large but not small cholangiocytes[89]. Chronic feeding of ursodeoxycholate and tauroursodeoxycholate to BDL rats inhibits secretin-stimulated ductal secretion in large cholangiocytes[62].

As evidence against the notion that small cholangiocytes may be primitive, undifferentiated cells that do not display secretory activity, recent studies have shown that in pathological conditions associated with damage of large cAMP-responsive ducts (e.g., after acute CCl_4 administration) (Figure 3)[50,51], small cholangiocytes transiently compensate for large cholangiocyte damage by *de novo* activation of secretory (including expression of secretin receptor and secretin-stimulated cAMP response)[50,51] and proliferative[50,51] (see below) activities. Following ANIT feeding and partial hepatectomy, small cholangiocytes proliferate and secrete by the *de novo* expression of secretin receptor and activation of cAMP response[47,52]. Since preliminary data and unpublished observations (Alpini, 2005) show that small rat and mouse cholangiocytes express receptors (ET_A, CCK-B/gastrin, α1-adrenergic, D2 dopaminergic, insulin, H1 histamine) signaling by activation of IP_3/Ca^{2+}/PKC[59,90], we propose that there is a secretory gradient in the intrahepatic biliary tree with small cholangiocytes secreting water and electrolytes by activation of the IP_3/Ca^{2+}/PKC pathway, whereas large cholangiocytes secrete bile by activation of the cAMP/PKA/CFTR/Cl^-/HCO_3^- exchanger[2,5,2,13,48,54].

PROLIFERATION AND APOPTOSIS

Cholangiocyte proliferation is coordinately regulated by a number of factors including gastrointestinal hormones/peptides, growth factors, cAMP and IP_3/Ca^{2+}/PKC pathways, nerves and bile acids[2,3,24,49,61,62,68,70-72,91-93]. Recent studies have shown that different sized cholangiocytes differentially proliferate or are damaged by apoptosis in response to injury, toxins, nerve resection and selected diets[2,26,48-52]. Following BDL, large but not small cholangiocytes proliferate with increases in basal and secretin-stimulated choleresis (Figure 3)[2,3,48]. We propose that large cholangiocytes selectively proliferate in response to BDL due to: (1) the predominant expression of VEGF in large compared to small cholangiocytes (Alpini *et al*, 2005, unpublished observation); and (2) the presence of the PBP mainly around large bile ducts, and less discernable around small bile ducts[44]. In support of this

concept, in rats with BDL proliferation of the peribiliary plexus occurs only around large ducts[44]. Furthermore, we have recently demonstrated[93] that neutralization of VEGF levels of large cholangiocytes (by administration of a neutralizing anti-VEGF antibodies) reduces cholangiocyte growth typical of BDL rats[6]. In support of the concept that PBP and VEGF play a role in the regulation of large cholangiocyte function, hepatic artery ligation in BDL rats is associated with: (1) the disappearance of the PBP; (2) increased apoptosis and impaired proliferation of large cholangiocytes; and (3) decreased cholangiocyte VEGF secretion[92]. The effects of hepatic artery ligation on PBP and large cholangiocyte function were prevented by chronic administration of r-VEGF-A that, by maintaining the integrity of the PBP and large cholangiocyte proliferation, prevents bile duct damage following ischemic injury[92].

A number of gastrointestinal hormones/peptides have been shown to regulate the differential proliferative response of small and large cholangiocytes. We have shown that cholangiocytes express α1, α2, β1 and β2 thyroid hormone receptors and that the chronic administration of the thyroid hormone agonist, 3, 3′, 5 L-tri-iodothyronine to BDL rats reduces *in vivo* the proliferation of large cholangiocytes[94], the only cholangiocyte subpopulation proliferating in this model[48]. In addition, in BDL rats we have shown that somatostatin inhibits the growth of large cholangiocytes by a decrease in cAMP levels[48]. Furthermore, gastrin inhibits large cholangiocyte proliferation in BDL rats by Ca^{2+}/PKC-dependent inhibition of cAMP levels[72].

We have demonstrated that ovariectomy in BDL female rats reduces the proliferation of large cholangiocytes and induces a decrease in the expression of α and β estrogen receptors[69]. We propose that estrogens play a role in the management of chronic cholestatic liver diseases.

Recent studies have shown that nerves regulate the differential proliferative response of intrahepatic ducts. We have shown that the activation of serotonin 1 A and 1 B receptors in cholangiocytes leads to the inhibition of large cholangiocyte proliferation in BDL rats[67]. Serotonin inhibition of large cholangiocyte proliferation was associated with activation of the IP_3/Ca^{2+}/PKC signaling pathway and the consequent inhibition of the cAMP/PKA/Src/ERK 1/2 pathway[67]. Since cholangiocytes secrete serotonin, we propose that serotonin limits the growth of intrahepatic bile ducts in the course of chronic cholestasis by an autocrine mechanism. Similarly, we have shown that cholangiocytes secrete NGF and that NGF secretion increases in proliferating BDL cholangiocytes compared to normal cholangiocytes[24]. *In vivo*, immunoneutralization of NGF (with an anti-NGF antibody) decreased large cholangiocyte proliferation[24]. The data suggest that NGF regulates cholangiocyte proliferation by an autocrine mechanism.

We have demonstrated that sensory innervation via α-calcitonin gene related peptide (α-CGRP) plays a role in adaptive proliferative responses of large cholangiocytes during cholestasis following BDL[95]. Specifically, we have shown that small and large murine cholangiocytes express the CGRP receptor components (calcitonin like receptor or CLR, receptor component protein or RCP and receptor activity modifying protein or RAMP1)[95]. Large, but not small, cholangiocytes proliferate in response to α-CGRP, proliferation that was blocked by CGRP[8-37], α-CGRP receptor antagonist[96]. α-CGRP stimulation of large cholangiocyte proliferation was associated with increased cAMP levels and phosphorylation of PKA and p38[96]. We observed a decrease in the number of proliferating large cholangiocytes in BDL knock-out mice (lacking α-CGRP) compared to BDL wild-type mice[95].

The role of the second messenger, cAMP, in the regulation of hepatic cell proliferation has been demonstrated in a number of animal models that stimulate hepatocyte and cholangiocyte proliferation via cAMP dependent mechanisms[26,50-52,54,97,98]. Following partial hepatectomy, there is an increase in intracellular cAMP levels in regenerating hepatocytes[99] and cholangiocytes[52]. Activation of $G\alpha_s$ coupled receptors leads to activation of adenylyl cyclase and increased cAMP levels, whereas activation of $G\alpha_i$ coupled receptors results in inhibition of AC activity and lowered intracellular cAMP levels[100]. cAMP response elements mediating transcriptional activation in response to increased intracellular cAMP levels have been identified[101]. In support of these findings, we have shown that chronic administration of forskolin to normal rats increased cAMP levels and the proliferation of large but not small cholangiocytes compared to rats receiving saline[70]. In purified cholangiocytes, forskolin increased large (but not small) cholangiocyte proliferation[70], which was blocked by Rp-cAMPs (a PKA inhibitor)[74], PP2 (a Src inhibitor)[102] and PD98059 (a MEK inhibitor)[103]. The effects of forskolin on large cholangiocyte proliferation were associated with increased phosphorylation of PKA, Src Tyr 139 and ERK1/2[70]. Maintenance of cAMP levels by forskolin administration prevents the effects of vagotomy on large cholangiocyte apoptosis (activation) and proliferation (inhibition)[26].

The acute administration of CCl_4 to normal and BDL rats induces decreased cAMP levels and loss of function of large cholangiocytes at d 2 and transient elevation of cAMP levels in small cholangiocytes[50,51]. In these models, small cholangiocytes *de novo* express secretin receptors, a key component of the biliary proliferative and secretory mechanisms, suggesting that intracellular cAMP plays a key role in the: (1) *de novo* expression of large cholangiocyte phenotypes by small cholangiocytes (to compensate for loss of large cholangiocyte function); and (2) perhaps the differentiation of small cholangiocytes towards a cholangiocyte subpopulation that has the capacity to secrete and proliferate by cAMP-dependent pathway[50,51].

Following partial hepatectomy, both small and large cholangiocytes proliferate and participate in the regeneration of the intrahepatic biliary epithelium[52]. A single gavage dose of CCl_4 to normal and BDL rats induces damage of large, cAMP-responsive cholangiocytes, whereas small cholangiocytes (resistant to CCl_4) *de novo* proliferate and secrete (by the activation of the secretin receptor and secretin-stimulated cAMP levels) to compensate for the damage and loss of functional activity of large cholangiocytes[50,51]. The differential resistance of small and large cholangiocytes to CCl_4 is presumably due to the presence

of cytochrome P4502E1 (the enzyme that converts CCl_4 to its radicals)[104] in large but not small cholangiocytes[50,51]. Chronic administration of the toxin, ANIT, induces proliferation of both small and large cholangiocytes, proliferation that (in contrast to other models including BDL)[26] was associated with enhanced apoptosis[47]. We propose that following ANIT or CCl_4 feeding, the proliferation of small cholangiocytes may be due to the presence of cholangiocyte apoptosis in these models[47,50,51]. We also propose that the lack of small cholangiocyte proliferation in BDL rats may be due to the absence of cholangiocyte apoptosis in this model[26]. Similar to what is observed following acute CCl_4 administration[50], the differential responses of small and large cholangiocytes to liver injury/toxins may be due to differential expression of other enzymes/proteins in small and large cholangiocytes. In support of this concept, phase I or mixed-function oxygenase enzymes (e.g., microsomal cytochrome P-450, aminopyrine-N-demethylases, G-6-PO_4, and NADPH cytochrome C reductase) and phase II or glutathione redox cycle enzymes (e.g., GSH-peroxidase, UDP-glucuronosyltransferase, and glutathione-S-transferase) drug-metabolizing enzymes are heterogeneously expressed by cholangiocytes[50,81,105]. Similarly, since small murine cholangiocytes express annexin-V[106] (that regulates cell apoptosis)[107], this finding may explain partly why small ducts are more resistant than large ducts to some hepatic injury/toxins[50, 51]. In support of this concept, recent studies have shown that bcl-2 (an anti-apoptotic protein)[108] is expressed by small bile ducts in normal human liver and human liver with cirrhosis and focal nodular hyperplasia[109], a finding that may also explain partly the greater resistance of small cholangiocytes to damage[3,50,51].

In vitro treatment of normal cholangiocytes with taurocholate and taurolithocholate increases the proliferation of large but not small cholangiocytes[89]. Chronic feeding of taurocholate and taurolithocholate to normal rats induces the *de novo* expression of ASBT and activation of proliferation of small cholangiocytes, which do not constitutively express ASBT and are mitotically quiescent, and increases the proliferation of large cholangiocytes[49]. Prolonged feeding of ursodeoxycholate and tauroursodeoxycholate to BDL rats reduces the growth of large cholangiocytes[62] that selectively proliferate in this hyperplastic model[48]. Furthermore, depletion of endogenous bile acids reduced large cholangiocyte proliferation compared with BDL rats[91]. Re-infusion of taurocholate to bile acid-depleted rats prevented the decrease in cholangiocyte proliferation that was maintained at levels similar to those of BDL rats[91].

Histamine, an aminergic neurotransmitter, regulates many pathophysiological functions. Four G-protein coupled histamine receptors (H_1, H_2, H_3 and H_4) exist[110]. While H_1 histamine receptors act via $G\alpha_q$ mobilizing $[Ca^{2+}]_i$[111], activation of H_2 histamine receptors is modulated by $G\alpha_s$ proteins, coupled to adenylyl cyclase[112]. H_3 and H_4 histamine receptors couple to $G\alpha_{i/o}$ proteins that inhibit adenylyl cyclase[113]. Based upon our preliminary data, we propose a model in which the overall outcome of histamine on cholangiocyte growth is represented by a balance between its stimulatory (by activation of H1 and H2 histamine receptors)[90,114] and its inhibitory (by activation of H3 and H4 histamine receptors)[115, 116] actions on small and large cholangiocyte proliferation. Specifically, we have shown that small but not large mouse cholangiocytes: (1) express the H_1 histamine receptors and the calcium-dependent CaMK I (but not II or IV) protein kinase; and (2) proliferate in response to H_1 histamine receptor agonists, proliferation that was blocked by BAPTA/AM, Gö6976 and W-7, a CAMK inhibitor[117]. IP_3 (but not cAMP) levels were increased in small cholangiocytes treated with HTMT dimaleate. Chronic administration of the specific H3/H4R agonist (RAMH) to BDL rats decreased large cholangiocyte proliferation and cAMP levels compared to BDL rats treated with NaCl[115,116]. This inhibition is mediated through negative regulation of the cAMP-dependent PKA/ERK1/2 pathway[115,116].

The mechanisms by which different sized ducts proliferate or are damaged in response to various liver injury/toxins (e.g., BDL, partial hepatectomy, vagotomy, feeding of ANIT, bile acids or CCl_4)[3,26,47-52] are unclear. Furthermore, the pathophysiology of small cholangiocytes is undefined in these models. Based upon preliminary data and unpublished observations from our laboratory, we propose that neural/hormonal-dependent (cholinergic and adrenergic) activation of the Ca^{2+}-dependent NFAT (Nuclear Factor of Activated T-lymphocytes) stimulates the proliferative response of small cholangiocytes, whereas neural/hormonal-dependent activation of the cAMP-dependent CREB stimulates the proliferation of large cholangiocytes. NFAT is a ubiquitous transcription factor that was initially described in T-lymphocytes. Five isoforms of NFAT have been identified. Four of these isoforms (NFATc1 to c4) are regulated by Ca^{2+} signaling[118]. Preliminary data shows that Ca^{2+}-dependent activation of NFATc1/c4 stimulates the proliferation of small cholangiocytes after CCl_4-induced damage of cAMP-responsive large bile ducts[119]. Specifically, we have shown that small but not large normal rat cholangiocytes express the NFAT isoforms, NFAT c1 and c4[119]. CCl_4 both *in vivo* and *in vitro* increased small cholangiocyte proliferation that was blocked by BAPTA/AM and 11R-VIVIT (NFAT inhibitor peptide)[120]. Furthermore, unpublished data from our laboratory show that the *de novo* growth of small cholangiocytes is regulated via adrenergic stimulation of Ca^{2+}-dependent activation of NFATc1/c4 (Ca^{2+}/calcineurin) and Sp1 (Ca^{2+}/PKC). NFAT and Sp1 cooperatively interact to regulate proliferative phenotypes in other cell types[121].

Recent studies have shown that bile acids have cytoprotective effects against apoptosis in large cholangiocytes. Feeding of taurocholate to BDL rats (treated with a single dose of CCl_4) prevents CCl_4-induced damage of large cholangiocytes, whereas small cholangiocytes (which are *de novo* activated following CCl_4-induced damage of large ducts)[50,51] remained mitotically dormant and unresponsive to secretin (Figure 3)[122]. *In vitro*, taurocholate prevented the inhibitory effects of CCl_4 on apoptotic, proliferative and secretory capacity of large BDL cholangiocytes[122]. The protective effects of taurocholate against CCl_4-induced damage of large BDL cholangiocytes are due to the

activation of PI3-K and AKT expression[122]. Furthermore, feeding of taurocholate to BDL + vagotomy rats prevented vagotomy activation of large cholangiocyte apoptosis and inhibition of large cholangiocyte growth[123], effects that were abolished by wortmannin, a PI3-K inhibitor[124]. Functional ASBT expression as well as phosphorylation of Akt were reduced by vagotomy but restored by taurocholate feeding[123]. Chronic feeding of taurocholate prevented the increase in cholangiocyte apoptosis and the damage of large cholangiocyte proliferation induced by adrenergic denervation by 6-OHDA administration[125]. Taurocholate effects are mediated by the PI3K pathway, since the simultaneous administration of wortmannin reverses such effects[125]. In addition, the feeding of ursodeoxycholate and tauroursodeoxycholate to BDL + vagotomy rats prevented the activation of apoptosis and the loss of proliferation of large cholangiocytes observed in this model[126]. In this study[126], the protective effects of these two bile acids were neutralized by the simultaneous administration of BAPTA/AM (an intracellular Ca^{2+} chelator)[72] or Gö6976 (a PKC inhibitor)[65]. Both ursodeoxycholate and tauroursodeoxycholate increased IP_3 and Ca^{2+} levels, together with enhanced phosphorylation of PKC-α[126]. The data suggests that bile acids are important in modulating large cholangiocyte proliferation in denervated livers.

HETEROGENEITY IN CHOLANGIOPATHIES

Chronic cholestatic liver diseases (cholangiopathies), which target intrahepatic and extrahepatic bile ducts, are characterized by the coexistence of cholangiocyte growth/apoptosis, inflammation and fibrosis[3,127]. Cholangiopathies differentially target the biliary epithelium with heterogeneous proliferative and apoptotic responses of different sized ducts[3,47,50,128-130]. Primary biliary cirrhosis is characterized by the selective proliferation/loss of small interlobular bile ducts[3,131]. Some studies demonstrated that damage of interlobular bile ducts is immune mediated[3,132]. The origin of primary sclerosing cholangitis (PSC), which is associated with inflammation and fibrosis of bile ducts, originates from multiple factors including autoimmune, bacterial, congenital, drug, or viral agents[3,73]. PSC affects mainly extrahepatic and interlobular or septal bile ducts although smaller bile ducts can be affected[3,73]. Patients with small duct PSC seem to have a good prognosis in terms of survival and development of cholangiocarcinoma[133]. Cholangiocarcinoma occurs frequently in patients with PSC and targets mainly the major bile duct bifurcation[3,134]. Peripheral cholangiocarcinoma occur within the liver rather than within large bile ducts may arise from small bile ducts[3,134]. Mutations in the CFTR gene are responsible for causing the human biliary disease, cystic fibrosis, due to defective transport of water and chloride presumably by large cholangiocytes expressing CFTR[135]. Our previous studies in rodent liver has shown that CFTR is expressed principally in large cholangiocytes and in bile ducts greater than 15 μM diameter[12,13] but in studies of human liver of cystic fibrosis patients, CFTR was expressed in both large and small ducts[136].

Defective chloride transport and chloridemediated bile secretion by large cholangiocytes may be responsible for the reduced fluidity and alkalinity of bile, leading to bile duct damage. Ca^{2+}-dependent Cl^- channels[137,138] (presumably expressed by both small and large cholangiocytes) may be able to secrete bile, thus compensating for loss of CFTR functional activity of CFTR in large cholangiocytes[54]. In polycystic kidney liver disease (PKLD), the genetic defect results in the growth of multiple epithelial cysts within the renal, liver parenchyma and intrahepatic bile ducts[139]. The disease targets presumably large bile ducts since the cystic ductal cells also secrete Cl^- and HCO_3^- (as normal large cholangiocytes)[2,3,54,71,73] but the secretion is diminished, likely due to reduced Cl^-/HCO_3^- exchanger activity in cystic ductal cells as compared with normal cholangiocytes[139]. Biliary atresia, which is the most common reason of cholestasis in infants and children, is a destructive, inflammatory process of the extrahepatic bile ducts but as the disease progresses smaller intrahepatic bile ducts are also involved[140]. The pathogenesis of biliary atresia is unknown but infections or toxic agents combined with genetic/immunologic susceptibility have been proposed[3, 141, 142].

SUMMARY

In this review, we have summarized the findings demonstrating that the intrahepatic biliary epithelium is heterogeneous regarding: (1) morphological characteristics, vascularization and innervation; (2) secretory activity in response to gastrointestinal hormones/peptides, nerve receptor agonists and bile salts; and (3) apoptotic and proliferative responses to liver injury/toxins and gastrointestinal hormones/peptides. Specifically, the intrahepatic biliary epithelium is formed by bile ducts of different sizes with small ducts lined by small cholangiocytes, whereas larger ducts are lined by larger cholangiocytes[12-14]. Following a general background on cholangiocyte functions, we discussed the *in vivo* and *in vitro* experimental models that allowed us to demonstrate that the biliary epithelium is morphologically and functionally heterogeneous. Following a brief review on the heterogeneous distribution of non-transport related proteins, we discussed the secretory functions of small and large cholangiocytes. While large cholangiocytes secrete water and electrolytes[12,13,48] by changes in cAMP/PKA/CFTR/Cl^-/HCO_3^-, small cholangiocytes may secrete bile by a transduction pathway (different from that observed in large cholangiocytes)[12,13,48] involving activation of IP_3/Ca^{2+}/PKC. We have presented data demonstrating that small and large cholangiocytes differentially proliferate or are damaged in response to liver injury/toxins. Small and large ducts also differ regarding the proliferative and apoptotic responses to liver injury/toxins[2,71,73]. We propose that activation of the Ca^{2+}-dependent NFAT stimulates the proliferation of small cholangiocytes, whereas neural/hormonal-dependent activation of the cAMP-dependent CREB stimulates the proliferation of large cholangiocytes. In the last part of the review, we have briefly outlined the heterogeneity of the biliary epithelium in relationship to chronic cholestatic liver diseases targeting different sized ducts.

FUTURE PERSPECTIVES

The concept that the biliary epithelium is functionally heterogeneous is clinically relevant since in chronic cholestatic liver diseases cholangiocyte proliferation/damage is an event restricted to a specific duct size. Further studies are needed for understanding the pathophysiology of small cholangiocytes in the overall contribution of the functions of the biliary epithelium. However, some preliminary studies from our laboratory suggest that small cholangiocytes secrete bile (by a IP_3/Ca^{2+}/PKC-dependent mechanism) and proliferate by activation of the Ca^{2+}-dependent transcription factor, NFAT. Further studies are necessary to evaluate the role of the nervous system in the regulation of the heterogeneous secretory, apoptotic and proliferative responses of different sized bile ducts to gastrointestinal hormones, injury/toxins and viruses. Since PBP proliferation is observed only in large proliferating cholangiocytes from BDL rats, we propose that blood supply and circulating factors (e.g., vascular endothelial growth factor and placental growth factor) may be important in the regulation of the heterogeneous response of cholangiocytes to liver injury/toxins.

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Estrogens and the pathophysiology of the biliary tree

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Abstract

The scientific framework concerning estrogen effects on different tissues has expanded enormously during the last decades, when estrogen receptor (ER) subtypes were identified. Estrogens are not only essential for the female reproductive system, but they also control fundamental functions in other tissues including the cardiovascular system, bone, brain and liver. Recently, estrogens have been shown to target the biliary tree, where they modulate the proliferative and secretory activities of cholangiocytes, the epithelial cells lining bile ducts. By acting on both estrogen receptors (ER-α) and (ER-β) subtypes, and by activating either genomic or non-genomic pathways, estrogens play a key role in the complex loop of growth factors and cytokines, which modulates the proliferative response of cholangiocytes to damage. Specifically, estrogens activate intracellular signalling cascades [ERK1/2 (extracellular regulated kinases 1/2, PI3- kinase/AKT (phosphatidylinositol-3' kinase/AKT)] typical of growth factors such as insulin like growth factor (IGF1), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF), thus potentiating their action. In addition, estrogens stimulate the secretion of different growth factors in proliferating cholangiocytes. This review specifically deals with the recent advances related to the role and mechanisms by which estrogens modulate cholangiocyte functions in normal and pathological conditions.

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Key words: Estrogens; Cholangiocytes; IGF1; Proliferation; APDKD; PBC; Cholangiocarcinoma; SERMs

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INTRODUCTION

The intrahepatic biliary tree is a complex three-dimensional network of interconnected ducts, which starts at the level of canals of Hering, continues into intrahepatic ducts of increasing diameter, and ends at the level of main extrahepatic bile ducts[1-3]. Recent studies demonstrated that the intrahepatic biliary tree plays a critical role in many liver functions including bile formation, regeneration, injury repair, fibrosis, angiogenesis and regulation of blood flow[4,5]. Most of these events are regulated by a complex network of neuropeptides, hormones, cytokines and growth factors, which target cholangiocytes[6,7], the epithelial cells lining the biliary tree. Cholangiocytes are the primary target of damage in a group of chronic cholestatic liver diseases, named cholangiopathies, with high social and economic impact due to their high prevalence and morbidity[8]. Altogether these pathologies represent a main indication for liver transplantation and, in fact, in 2003, 10% of OLT in the USA had as an indication PBC (primary biliary cirrhosis) and PSC (primary sclerosing cholangitis).

Although very heterogeneous in the etiology and pathogenesis (autoimmune, infectious, vascular, drug-

induced and cryptogenic)[8], the cholangiopathies share common pathological features such as the injury of intrahepatic bile ducts, the proliferation of residual ducts and the intralobular cholestasis[9]. In addition, most of these pathologies evolve towards ductopenia that represents the terminal stage of the disease[9]. For this reason, cholangiopathies have been also classified as vanishing bile duct syndromes[8,9]. In the early stage of the diseases, the disappearance of intrahepatic ducts is balanced by the proliferation of the residual bile ducts[10], which tends to compensate for the anatomical and functional loss of damaged ducts. In fact, proliferating bile ducts display enhanced secretory activities aimed to compensate for the impaired secretion of injured ducts[11]. Thus, the course of these diseases is characterized by a balance between damage (loss) of bile ducts and compensatory proliferation of the residual ducts[10]. In the terminal decompensated stages, the inefficacy of proliferation to balance for the loss of intrahepatic bile ducts leads to ductopenia, and thus to the clinical manifestations of overt cholestasis[12]. Therefore, the design of a therapeutic strategy aimed at supporting an efficacious cholangiocyte proliferation could delay the progression toward ductopenia and this represents a challenge for the future. For these reasons, during the last few years, mechanisms and agents involved in the modulation of cholangiocyte proliferation have been extensively evaluated, especially at the experimental level[5-7]. To this latter regard, the experimental model of the common bile duct ligated (BDL) rat was very helpful since the typical and selective cholangiocyte proliferation that follows BDL induces a tremendous expansion of cholangiocyte mass (30% of parenchymal hepatic cells with respect to 2% in normal) thus facilitating the study of cholangiocyte pathophysiology. Thanks to recent studies, we now know that cholangiocyte proliferation is regulated by several growth factors, hormones, neuropeptides and bile salts[5-7]. One of the objectives of these studies was related to estrogens[13-16] since, for many years, we have become aware that the clinical appearance and progression of different cholangiopathies are influenced by gender and changes of estrogen status in the body[17]. Nevertheless, only in the last few years we have learned that estrogens and their receptors influence the pathophysiology of cholangiocytes and that this mainly occurs during experimental and human conditions characterized by cholangiocyte injury and proliferation[13-16]. Estrogens have been considered for many years to play a role in the development and progression of pathologies involving the biliary tree[17,18]. This hypothesis was based on a number of clinical observations including: (1) PBC, the most prevalent acquired cholangiopathy, specifically affects females[17,19] with a clinical presentation typically occurring during the peri- and post-menopausal period[19-23]; (2) endocrine dysfunctions are frequent in PBC[17,19,21] including an increased incidence of menstrual disturbance and hysterectomy[19,21] and, the high incidence of post-menopausal osteoporosis that is a sign of estrogenic deficiency[24] and that is corrected by estrogen replacement therapy[25]; (3) chronic hepatic rejection leading to ductopenia more frequently from male donor into female recipient[26]; (4) in Turner syndrome, in which liver morphology resembles that of a newborn liver, bile duct pathology is frequent and is improved by estrogen replacement treatment[27]; (4) the progression of polycystic liver disease is significantly influenced by female sex, pregnancies and estrogen replacement treatment. In addition, marked alterations of estrogen hepatic metabolism occur in cholestasis[28], which is one of the hallmarks of cholangiopathies, leading to enhanced estradiol serum levels[28], which could influence disease progression. In spite of all these clinical considerations, the role and mechanism by which estrogens modulate cholangiocyte functions have been explored only in the last few years at both experimental and clinical levels[13-16].

EXPERIMENTAL STUDIES

In 1896, the British physician George Beatson, firstly showed that oophorectomy induces the regression of mammary tumors in a subset of premenopausal patients. Since then, a variety of clinical and epidemiological observations have further substantiated the involvement of estrogens as inducers of growth and differentiation of target cells expressing estrogen receptors (ERs)[29]. Estrogens exert their "trophic" action on many different target organs, among which is the liver[30], where they modulate growth and repair, contributing to neonatal liver growth and regeneration after injury in adults[29]. In injured liver, ERs expression increases with respect to normal liver where ERs are expressed at a very low level[30]. Furthermore, chronic administration of estrogens for pharmacological purposes, induces an enlargement of liver mass[31,32] and, after partial hepatectomy, ERs expression in hepatocytes increases and localizes predominantly into the nucleus with subsequent transcription of genes involved in proliferation, thus favoring the restoration of a normal liver mass[32]. Recent findings indicate that estrogens also target the biliary tree playing an important role in modulating cholangiocyte proliferation[13]. Even if ER-α and β subtypes are also expressed by normal rat cholangiocytes[14], their expression (especially ER-β) markedly increases in proliferating cholangiocytes of BDL rats, whereas hepatocytes, which do not proliferate after BDL, display a ten-fold decrease of ERs protein expression[13]. During cholestasis (after BDL) estrogen serum levels are increased[20, 28] and this could play a role in sustaining cholangiocyte proliferation by a complex loop of agent among which estrogens may act either directly or potentiating the effects of different growth factors. In addition ER antagonists (tamoxifen, Ici 182,780) block the BDL-induced increase in intrahepatic bile duct mass by impairing cholangiocyte proliferation and enhancing apoptosis[13-16]. Similarly, in hepatocellular carcinoma[33] and breast cancer cells, tamoxifen counteracts estrogen mitogenic effect either by antagonizing estrogens or by inducing apoptosis-related genes[33]. In cholangiocarcinoma cell lines[33-35], tamoxifen induces apoptosis mainly by the activation of Fas receptor/Fas ligand pathway. Interestingly, the involvement of Fas antigen has been also demonstrated in PBC and PSC[36,37], where the imbalance between proliferation and apoptosis could play an important role in disease pathogenesis and progression. All these findings suggest that, in BDL rats, estrogen antagonists could block cholangiocyte proliferation and activate apoptosis by a Fas dependent mechanism, even if other

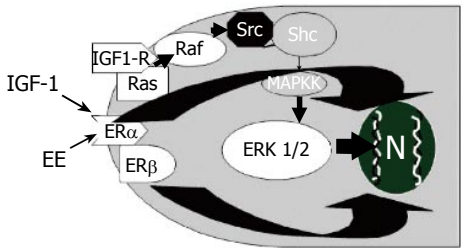


Figure 1 Additive effect of estrogens (EE) and insulin like growth factor-1 (IGF1) in the modulation of cholangiocyte proliferation. EE and IGF1 play additive effects on cholangiocyte proliferation by acting at both receptor and post-receptor levels IGF1 and EE induce, in cholangiocytes, additive increase of phosphorylated ERK1/2.

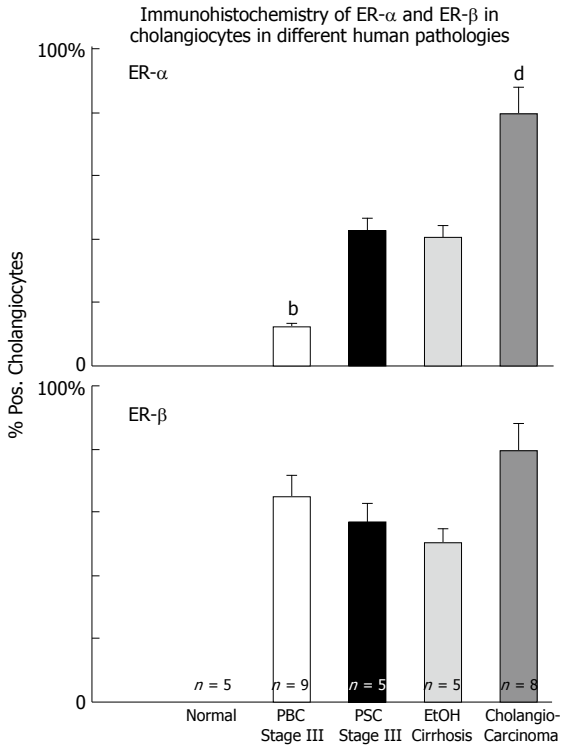


Figure 2 Immunohistochemistry of ER-α and ER-β in cholangiocytes in different human pathologies, $^{b}P < 0.01$, $^{d}P < 0.01$ *vs* other columns.

mechanisms of activation, especially for tamoxifen, cannot be excluded[32,33]. The concept that estrogens are positive modulators of cholangiocyte proliferation is further supported by the fact that, *in vitro*[13] 17β-estradiol significantly increases both proliferating cell nuclear antigen (PCNA) protein expression and 3H-thymidine incorporation into DNA and that two distinct ER antagonists (tamoxifen, Ici 182,780) block 17β-estradiol effects[13]. In addition, ovariectomy prevents cholangiocyte proliferation in BDL rats[15] and causes a 3-fold decrease of bile duct mass with respect to controls, in association with a 2.5-fold lower expression of Er-α and a 35-fold lower expression of ER-β[15]. When 17β-estradiol was administered in BDL + ovariectomy rats, bile duct proliferation was restored and ER expression in cholangiocytes returned similar to controls[15]. The proliferative effects of estrogens in target cells expressing ER can involve both a direct genomic pathway and an indirect nongenomic pathway where extracellular-regulated kinases (ERK1/2 = p42/p44 MAP kinases) are involved[38]. This has been also demonstrated in cholangiocyte proliferation occurring after BDL where estrogen administration increases the expression of phosphorylated ERK1/2[39]. In contrast, tamoxifen decreases phosphorylated ERK1/2 in association with impaired cholangiocyte proliferation[39]. The Src/Shc/ERK1/2 cascade is typically activated by several growth factors[40], thus suggesting that estrogens could potentiate the effects of growth factors by sharing similar intracellular signaling pathways. Consistently, in cholangiocytes, estrogens exert additive effect with NGF and IGF1[41,42] on modulating proliferation. As far as IGF1 is concerned, the cooperation with estrogens (Figure 1) occurs at both receptor and post-receptor level, since estrogens and IGF-1 increase phosphorylation of IGF1-R, ERK and AKT, whereas the interplay between NGF and estrogens occurs mainly at the level of PI3-kinase[42]. Another growth factor able to sustain cholangiocyte proliferation is VEGF. Very recently, we showed that estrogens induce VEGF synthesis and release in isolated cholangiocytes and this was markedly amplified in proliferating cholangiocytes[43]. This finding is very attractive since under estrogen stimulation, proliferating cholangiocytes can modulate, *via* VEGF release, their vascular supply whose adaptive response is critical for sustaining the enhanced functional and nutritional demands of the proliferating biliary tree[43]. Moreover, VEGF released in the peribiliary plexus by proliferating cholangiocytes following cholestasis could also favour the survival and repair of hepatocytes[43].

HUMAN STUDIES

Cholangiocytes of normal liver do not express ERs at the immunohistochemical analysis[16] but they stain positive for ER-α and -β in several pathological conditions including PBC[16], polycystic liver disease[44] and cholangiocarcinoma[45] (Figure 2). All these conditions are characterized by reactive or neoplastic cholangiocyte proliferation, suggesting that estrogens and their receptors may play a role in modulating the proliferative activities of cholangiocytes and therefore the course of these diseases.

ESTROGENS AND PBC

PBC is a chronic cholestatic liver disease, which represents the most frequent acquired cholangiopathy[46]. Actually, PBC is considered an autoimmune disease with a sexual dimorphism[46]. The disease, in fact, predominantly affects females (10:1 female/male ratio) with a typical clinical presentation occurring during the peri- and post-menopausal period[19,20,46]. The thought that estrogens can influence the clinical course of PBC, as for most autoimmune disorders, came from several studies showing that estrogens are able to modulate the humoral and cellular immune response[47] through the inhibition of Th1 pro-inflammatory cytokines (IL-12, TNF-alpha and IFN-gamma)[48]. By switching the immunological response toward the Th2 profile, estrogens stimulate the production of Th2 anti-inflammatory cytokines (such as IL-10, IL-4 and TGF-beta) thus potentiating the anti-inflammatory response[48]. In addition, estrogens can prevent oxidative stress in hepatocytes[49,50], which

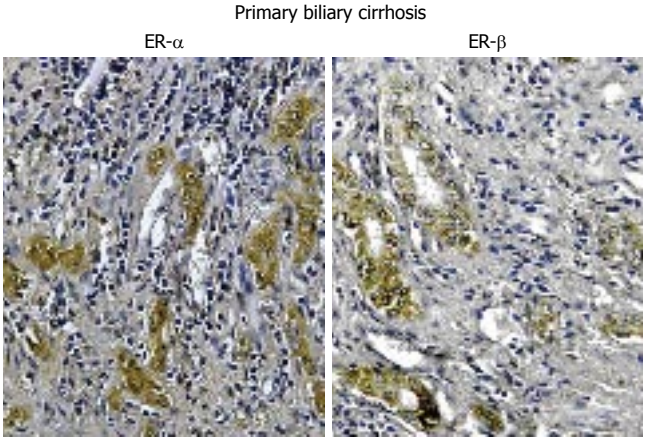


Figure 3 Immunohistochemistry for ER-α and ER-β. Biopsies of human primary biliary cirrhosis showing an intense positivity for both ER-α and ER-β in the proliferating bile ducts. Orig. magn., x 20.

are injured by cholestasis[12]. In spite of all these considerations, recent findings suggest that estrogens may influence the course of PBC by directly modulating the pathophysiology of cholangiocytes[13-16], which are the primary target of damage in this disease.

In PBC, such as in other chronic cholestatic conditions, estrogen serum levels are increased as a consequence of impaired hepatic metabolism and biliary excretion of estrogens and their metabolites[28]. Until recently, the high serum concentration of estrogens occurring in PBC patients was considered to have a negative effect on disease progression. For a long time, in fact, estrogens have been used in rodents as an experimental model of intrahepatic cholestasis[28]. Therefore, estrogen administration has been avoided in PBC patients. Recently, however, estrogen replacement therapy, as osteoporosis treatment, has been shown to be safe in PBC patients[51]. Thereafter, other studies[25] with the same end-points (i.e. osteoporosis treatment) indicate an improvement of liver serum enzymes (including cholestasis enzymes) during estrogen administration in PBC patients. These clinical studies allowed us to rule out the concept that administration of estrogens in PBC patients exerts deleterious effects on the liver but, on the contrary, suggest that they can improve liver function other than ameliorating bone mineral density.

Another old concept is the existence of a condition of estrogenic dysfunction in PBC[19-21]. This concept was based on the high frequency of menstrual abnormalities and hysterectomy[21], on the increased risk of breast cancer[22,23] and other malignancies[20], attributed to the increased estrogen serum levels, and increased prevalence of post-menopausal osteoporosis[24] which is corrected by estrogen replacement. However, these observations are still the object of controversies[21-23,52,53]. The incidence of extrahepatic malignancies[54], for example, in a cohort of Italian PBC patients, was shown to be lower with respect to the general population and to what was shown for American and Northern European PBC patients. In the same study, the incidence of breast cancer in PBC was comparable to that expected in the general population[54]. Also for metabolic bone disease associated with the progression of PBC, definitive data are still lacking. Recent studies[55,56] indicate that PBC is a risk condition *per se* for post-menopausal bone disease (osteoporosis and osteopenia), which occurs with the same incidence compared to other chronic liver diseases. Therefore, advanced liver disease, rather that PBC *per se*, represents a condition favouring the development of osteoporosis[55,56].

Nevertheless, recent studies indicate that estrogens play a role in the pathophysiology of PBC since both experimental and clinical studies indicate that they modulate cholangiocyte survival and death[13-16]. Cholangiocytes lining interlobular bile ducts of PBC patients, but not normal subjects, express both Er-α and -β subtypes, at the immunohistochemical analysis[16] (Figure 3). The ER expression varies according to different stages of disease and correlates with markers of proliferation (PCNA) and death (Tunel)[16]. ER-α expression increases from 1% of cholangiocytes in PBC stage I to 12 % in stage III while, ER-β is stably high (50%-65%) in all histological stages of PBC[16]. Interestingly, in stages I-III of PBC, ER-α expression correlates with and co-localizes with PCNA indicating that the expression of this receptor subtype is a typical feature of proliferating cholangiocytes[16]. Furthermore, in stage IV of PBC, when the maximal degree of ductopenia is reached, cholangiocytes are negative for ER-α and express the lowest PCNA/TUNEL ratio[16]. This raises the speculation of a relative proliferative deficiency of cholangiocytes in the terminal ductopenic stages of PBC, which is associated with the disappearance of ER-α. In addition, ER-α expression in cholangiocytes of PBC patients was markedly lower when compared with primary sclerosing cholangitis and alcoholic cirrhosis[16] (Figure 2), whose prevalence is higher in male sex[9], suggesting that the female predominance of PBC and the typical post-menopausal presentation could be related to a defect in proliferative response of cholangiocytes to estrogens. These findings could have important therapeutic implication, since the modulation of ERs could delay the progression of PBC toward ductopenia. During the last decade, estrogens have been involved in the pathogenesis of different diseases including atherosclerosis, Alzheimer disease, multiple sclerosis, Parkinson disease and obesity[57-61]. Estrogens, in fact, play biological activities in several organs[62] including the cardiovascular system, nervous system, digestive system (colon, liver) and "male" organs such as prostate. In target tissues, estrogens may exert opposite actions and heterogeneous effects, promoting the resistance to apoptotic damage, modulating reparative processes and controlling inflammation[63-65]. The expression pattern of ER subtypes is fundamental to evocate the different effects of estrogens[66]. ER-α, for example, is critical in estrogen induced protection against brain injury during stroke[66]. On the contrary, overexpression of ER-α has been linked with cancer development and progression in different organs[67]. The functions of ER-β are less known although recent findings suggest a protective effect against uncontrolled or neoplastic cell proliferation[68]. In our setting, the modulation of cholangiocyte proliferation exerted by estrogens and their receptors could open new therapeutic scenario suggesting appropriate trials aimed to evaluate the effects of ER selective modulators (SERMs) on PBC clinical expression. To this latter regard, preliminary clinical observations by us and

others[69,70] indicate that tamoxifen (mixed agonist/antagonist for ER-α and only antagonist for ER-β) improves biochemical parameters of cholestasis in PBC patients. We described three cases of PBC[69] that underwent tamoxifen treatment for breast cancer. In two patients, tamoxifen administration for approximately 18 mo, caused a marked decrease of alkaline phosphatase (70%-85%) and gamma-GT (60%-75%) serum levels and, in the third case, where tamoxifen was combined with UDCA, a strong reduction of liver enzymes was observed[69]. Moreover, during the observational period, none of these three patients developed complications of liver cirrhosis[69]. The positive effects of tamoxifen on the serum levels of cholestasis enzymes in PBC have been also described by Bassendine *et al* in two PBC patients who developed breast cancer and received tamoxifen, after surgical treatment[70]. However, other than acting as ER modulator in cholangiocytes, tamoxifen could improve cholestasis in PBC by additional mechanisms. Recently, in fact, tamoxifen has been shown to activate the human pregnane X receptor (PXR), a nuclear receptor linked with cytochrome p 450 function and considered to be relevant in the UDCA mechanism of action[71]. Therefore, tamoxifen could theoretically activate these promiscuous nuclear receptors thus improving UDCA effects[71]. Nevertheless, tamoxifen could have important side effects such as the increased risk of endometrial cancer when advised for disease lasting decades like PBC[72]. Second and third generation SERMs do not have these side effects and thus considered safer[73]. Our preliminary data (unpublished observations) indicates that raloxifene (a second generation SERM avoids the proliferative effects on reproductive tissues and which do not form adducts with DNA) causes biochemical improvement of cholestasis enzymes in PBC patients as shown for tamoxifen. Furthermore, it improves bone mineral density in PBC[74]. Thus a therapeutic strategy aimed to positively modulate estrogen proliferation during the early stages of PBC represents a challenge for the future, since cholangiocyte proliferation acts as a repair and compensatory mechanism and its stimulation is always associated with enhanced secretory activities[11]. Moreover, very recent studies elucidated that ER-α mediates many other "beneficial" effects of estrogen including immunoprotection in autoimmune disease[75]. ER-α activation, in fact, inhibits inflammatory gene expression by preventing NF-kappa B nuclear translocation[76]. Interestingly, through the ER-α, estrogens can positively modulate the GH/IGF-1 axis. This further supports a possible therapeutic role of ER-α positive modulators in cholangiopathies since we demonstrated that IGF- 1 and estrogens play additive effect on cholangiocyte proliferation (Figure 1)[42] and that the GH/IGF-1 axis plays a pivotal role in liver injury repair (unpublished observations). Consistently, IGF-1 replacement therapy shows clinical benefit in PBC and alcoholic cirrhotic patients[77].

ESTROGENS AND ADPKD

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most prevalent human genetic diseases with an incidence of 1:800 individuals[78]. Hepatic cysts are the most common extra-renal clinical manifestation of ADPKD and a significant source of morbidity[79]. That estrogens may have a role in the development and progression of hepatic cysts in ADPKD patients is suggested by a number of different clinical observations[82]. In ADPKD patients, the probability of developing hepatic cysts is higher in women with respect to men and among patients submitted to liver transplant for polycystic disease, more than 92% are women[80]. Furthermore, the number and size of hepatic cysts increase during post-menopausal hormonal replacement therapy[81] and nulliparae display less probability to develop hepatic cysts than pluriparae[82], where number and size of hepatic cysts correlate with the number of pregnancies[82]. As recently showed by us[44], interestingly, hepatic cysts are lined by cholangiocytes[83], that are different from normal biliary cells since they lack of both microvilli and primary cilia[84]. It is currently hypothesized that the lack of primary cilia, genetically determined, generates a cell phenotype characterized by hyperproliferating and hypersecretory properties. By acting on this cell phenotype, estrogens could sustain the enhanced proliferative and secretory activities, as experimentally shown in BDL rats[15], by acting either directly or potentiating the effects of growth factors. Recently, in fact, a number of different cytokines and growth factors (i.e. IL-8, IL6 and VEGF), markedly enriched in serum or hepatic cystic fluid, have been shown to promote the growth of hepatic cyst epithelium[85]. Immunohistochemical studies performed by our group (Alvaro, 2006, unpublished observations) showed that the epithelial surface of hepatic cysts of ADPKD patients display a marked and diffuse staining for ERs (mainly-β), IGF1 and IGF1-R. In addition, we demonstrated that[44] immortalized cell line (LCDE), derived from hepatic cyst epithelium of patients with ADPKD, also express ER-α and ER-β subtypes as well as IGF1 and IGF-R and that estrogens and IGF1 markedly stimulate the proliferation of this cell line by acting on their specific receptors[44]. Specific antagonists of ER (Ici 182,780) and an IGF1-R blocking antibody (α-IR3), in fact, inhibit the proliferating effect exerted by estrogens and IGF1 respectively[44] These recent findings may explain why estrogens induce the formation and worsen the progression of hepatic cysts in ADPKD patients. On the basis of our findings, the hepatic cyst epithelium of ADPKD patients could be considered a sort of estrogen responsive tissue, where the interaction between female sex hormones and growth factors, like IGF1 and VEGF, play a key role in modulating the pathophysiology. This could open new perspectives in pharmacological treatment of polycystic liver disease based on estrogen antagonism or on the use of selective SERMs.

ESTROGENS AND CHOLANGIOCARCINOMA

Cholangiocarcinoma is a tumor with increasing incidence and prevalence, that still represents a diagnostic and therapeutic challenge. It is one of the cancers with the worst prognosis, with a median 5-years survival rate of 7%-8% from the diagnosis and a median survival time of only seven months. The diagnosis is generally established late and

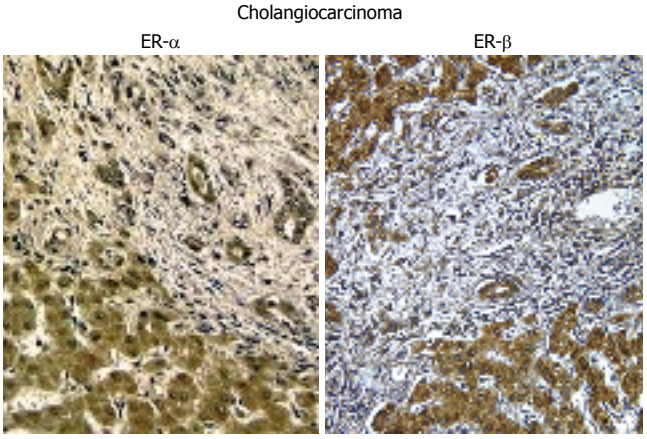


Figure 4 Immunohistochemistry for ER-α and ER-β. Biopsies of human cholangiocarcinoma showing an intense positivity for both ER-α and ER-β. Orig. magn., x 20.

surgery, when feasible, is the only effective treatment[86] Cholangiocarcinoma, in fact, shows a strong resistance to common chemotherapies and a treatment that slows the progression of the disease doesn't exist[86]. As for other cancers, an imbalance between proliferation and apoptosis leads to uncontrolled cell growth and expansion[37] We have recently shown, in human tissues and human cell lines, that estrogens play a key role in development and progression of cholangiocarcinoma[45]. Liver samples obtained from patients with intrahepatic cholangiocarcinoma were intensely positive for ER-α and ER-β subtypes (> 80% cholangiocarcinoma cells positive), while cholangiocytes of the normal liver were negative[45] (Figure 4). Interestingly, ER display both a cytoplasmic and nuclear staining, the latter being indicative of activated receptors[45]. When compared to benign cholangiocyte proliferation associated with non-neoplastic biliary disease (i.e. PBC, PSC), cholangiocarcinoma cells showed a higher expression of ER-α and increased ER-α/ER-β ratio[54] (Figure 2). This observation is in keeping with many different reports showing an increased ER-α/ER-β ratio in cancerous versus normal tissues, including ovary, prostate, colon and breast cancers[68]. In these tissues, primary events in neoplastic transformation and progression have been correlated with up-regulation ER-α and down-regulation of ER-β thus associating the function of ER-α subtype with a positive modulation of cell proliferation[68]. The opposite was suggested for ER-β[68]. Human intrahepatic cholangiocarcinoma also overexpress IGF1 and IGF1-R[45], another growth factor whose main source is liver and which is functionally linked with estrogens and their receptors[87,88]. IGF1 has been recently implicated in cancer development and progression since its serum concentrations correlate with an increased risk of breast, prostate, colorectal, pancreas and lung cancer[89-91] and, its receptor (IGF1R) is overexpressed in many tumor cell lines and in some human tumors. By studying a human intrahepatic cholangiocarcinoma cell line (HuH-28), similar to intrahepatic cholangiocarcinoma that over expresses ER-α, IGF1 and IGF1-R, we showed that IGF-1 and estrogens exert an additive proliferative effect[45] blocked by both ER antagonists (Ici 182,780) and IGF1-R blocking antibodies (α-IR3)[45]. Interestingly, IGF1-R seems to play a primary role also in the estrogen stimulation of neoplastic cell growth[45] since IGF1-R blocking antibody (α-IR3) partially inhibited the effects of 17β-estradiol and transfection of HuH-28 cell with IGF1-R antisense oligonucleotides caused a marked impairment of HuH-28 cells proliferation (90% decrease of PCNA expression)[45]. In estrogen sensitive tissues, a cross-talk between IGF1 and estrogen play a major role in the modulation of cell proliferation, where estrogens act at several points of the IGF signal transduction pathway[87]. Estrogens not only may regulate the expression of IGF1-R and IGF1-binding proteins but also that of crucial downstream proteins including IRS1, and the IGF1-R tyrosine kinase main substrate[88]. Finally, the signaling activated by estrogens and IGF-1 (Figure 1) may converge at different common transduction pathways, including ERK and phosphatidylinositol-3 kinase/Akt pathway[92,93], and this was observed in proliferating HuH-28 cells too. In addition, ER-α and IGF1-R, once activated coprecipitate and, their activation is potentiated by their coupling[45]. Nevertheless, estrogens can modulate other important mechanisms involved in cholangiocarcinoma growth such as COX-2[94]. These new evidences highlight the role of estrogens and IGF1 in regulating the growth of human cholangiocarcinoma and suggests that a therapeutic strategy based on the modulation of ERs and/or IGF1 system could be helpful for the management of this cancer.

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, recent studies support a relevant role of estrogens as modulators of benign (reactive) and neoplastic cholangiocyte proliferation[16,45,69,70]. In our hypothesis, estrogens act in concert with growth factors in sustaining the cholangiocyte proliferative machinery and in depressing apoptosis[41-43]. This may have a number of clinical implications for diseases involving the biliary epithelium, where cholangiocyte proliferation is a typical hallmark influencing disease progression[10]. As far as PBC is concerned, we believe that an impaired cholangiocyte response to estrogens characterizes the terminal ductopenic stages of the disease, where cholangiocyte proliferation is unable to balance for the enhanced apoptosis. On this basis, a SERM able to selectively stimulate the ER-α and conpemporarily depress the ER-β should be tested in controlled clinical trials. Furthermore, it is of interest that new generation SERMs, such as Idoxifene, play positive effects in controlling both the oxidative stress and fibrosis, which are foundamental processes in the progression of chronic cholestatic liver diseases[95-97]. As far as cholangiocarcinoma and ADPKD are concerned, pharmacological strategies aimed to inhibit estrogens binding to their receptors, to decrease their synthesis (by using aromatase inhibitors) and, finally, to down-regulate ERs protein levels (by using pure anti-estrogens such fulvestant), should be considered. Very recently an ER-X putative membrane associated estrogen receptor has been described in the brain and in the uterus[98]. Thus, further studies aimed to check the presence of this receptor subtype in cholangiocytes and to evaluate its role in the pathophysiology of the biliary tree are needed.

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Cholangiocytes and blood supply

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Abstract

The microvascular supply of the biliary tree, the peribiliary plexus (PBP), stems from the hepatic artery branches and flows into the hepatic sinusoids. A detailed three-dimensional study of the PBP has been performed by using the Scanning Electron Microscopy vascular corrosion casts (SEMvcc) technique. Considering that the PBP plays a fundamental role in supporting the secretory and absorptive functions of the biliary epithelium, their organization in either normalcy and pathology is explored. The normal liver shows the PBP arranged around extra- and intrahepatic biliary tree. In the small portal tract PBP was characterized by a single layer of capillaries which progressively continued with the extrahepatic PBP where it showed a more complex vascular network. After common duct ligation (BDL), progressive modifications of bile duct and PBP proliferation are observed. The PBP presents a three-dimensional network arranged around many bile ducts and appears as bundles of vessels, composed by capillaries of homogeneous diameter with a typical round mesh structure. The PBP network is easily distinguishable from the sinusoidal network which appears normal. Considering the enormous extension of the PBP during BDL, the possible role played by the Vascular Endothelial Growth Factor (VEGF) is evaluated. VEGF-A, VEGF-C and their related receptors appeared highly immunopositive in proliferating cholangiocytes of BDL rats. The administration of anti-VEGF-A or anti-VEGF-C antibodies to BDL rats as well as hepatic artery ligation induced a reduced bile duct mass. The administration of rVEGF-A to BDL hepatic artery ligated rats prevented the decrease of cholangiocyte proliferation and VEGF-A expression as compared to BDL control rats. These data suggest the role of arterial blood supply of the biliary tree in conditions of cholangiocyte proliferation, such as it occurs during chronic cholestasis. On the other hand, the role played by VEGF as a tool of cross-talk between cholangiocytes and PBP endothelial cells suggests that manipulation of VEGF release and function could represent a therapeutic strategy for human pathological conditions characterized by damage of hepatic artery or the biliary tree.

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Key words: Peribiliary plexus; Periportal plexus; Cholangiocytes

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INTRODUCTION

Cholangiocytes are currently the object of extensive investigation since recent evidence demonstrates their involvement in different processes (secretion, absorption, proliferation, neoangiogenesis), which are fundamental for

the liver pathophysiology[1-4]. The metabolic and functional demands of cholangiocytes are sustained by the hepatic artery which forms a complex network of vessels, the peribiliary plexus, around the biliary tree[5-9].

The central role of liver vascularization in hepatic functions and the importance of microcirculation for liver zonal organization were developed in the 17th century by Marcello Malpighi[10] who first described the "acinus", a hexagonal structure of the liver parenchyma that was comparable with a glandular unit of the liver. This description correlates with the current concept of a liver morpho-functional unit, also termed "acinus", described by Rappaport in 1952[11]. A number of studies on liver vascularization clarified the typical microvascular architecture that supplies the biliary tree. This typical microvascular network runs along the intra- and extrahepatic biliary tree and, as previously mentioned, has been termed peribiliary plexus (PBP)[12,13]. The structural study of either the intrahepatic and extrahepatic PBP in the liver under normal conditions has been the subject of a number of studies using both two-dimensional and three-dimensional approaches[5,12,14,15].

Although it is well known that the liver has a dual blood supply from the hepatic artery (HA) and the portal vein (PV), controversies still remain concerning the distribution of hepatic arterial blood flow. It is generally accepted that terminal branching of the HA opens into sinusoids, as demonstrated by injection of dyes and vascular casting [5,16-24]. The question that remains is if the HA provides arterial blood directly to sinusoids[16,20-22,23,25] or rather runs in the portal tract stroma, thus feeding the PBP and the periportal plexus (PPP) to finally arrive at the sinusoids[26-30]. The presence of a double feeding to the sinusoids from the PV, at a pressure of 6-7 cm H_2O, and from the HA, at a pressure of 12-25 cm H_2O, has generated many studies in order to explain the prevalence of flow from the PV[31]. Arterio-portal anastomoses are mainly observed in terminal portal tracts[23] but other studies deny the presence of real arterio-portal venous anastomoses[16,27,32,33].

A number of reports indicate that structural changes of the biliary tree, associated with experimental models of liver damage or human pathologies, are followed by substantial adaptive modifications of the PBP. At the experimental level, the bile duct-ligated (BDL) rat has been widely used as a model of typical and selective cholangiocyte proliferation[9,34,35]. This model is characterized by a dramatic increase of cholangiocyte number which reaches 30% of the total parenchymal hepatic cells compared with 2% in the normal liver [36]. In order to investigate the adaptive changes of hepatic microvascular organization associated with an increased mass of the biliary tree following BDL, our group has used the method of vascular corrosion casts observed with Scanning Electron Microscopy (SEM). This technique provides the ability to follow the tiniest vessels in three dimensions and to distinguish arterial, capillary and venous vessels by surface characteristics[7,13,15,26,37]. As detailed above, this technique demonstrates how the evolution of PBP expansion follows the proliferation of biliary tree suggesting a role of proliferating cholangiocytes in sustaining, through the secretion of angiogenic factors, and the adaptive proliferation of their vascular supply. To this regard, recent reports demonstrate that cholangiocytes are an important source of vascular endothelial growth factor (VEGF) in the damaged liver and that VEGF secreted by cholangiocytes may play role in driving the adaptive changes of the PBP, which accompany the proliferation of the biliary tree. Therefore, cross talk between cholangiocytes and endothelial cells could be fundamental in determining the response of the liver to cholestatic damage[38].

In this manuscript, we review recent studies related to the physiopathology of hepatic microvasculature in experimental models of liver damage and discuss the biological and clinical implications.

BLOOD SUPPLY

The microvasculature organization was investigated in normal or BDL rats by means of the Scanning Electron Microscopy Vascular Corrosion Casts (SEMvcc) technique[9,15,35]. The casts of normal livers showed an even distribution of sinusoids closely surrounding portal spaces. In larger portal spaces the hepatic artery (HA) branches were always evidently smaller than the portal ones. In smaller portal spaces the HA was often difficult to visualize[12,13,39,40]. The terminal branches of the HA are always represented by either the extra- and intrahepatic Peribiliary Plexus (PBP) or the periportal plexus (PPP). In very small portal spaces a small capillary, representing the terminal branch of a HA, can continue the course of the arteriole and eventually run into the sinusoids. It must be pointed out that it has all the characteristics of a capillary as far as diameter and endothelial imprints are concerned. This typical microvascular organization around the extrahepatic and intrahepatic biliary tree takes origin from the HA[15]. Hepatic artery branches paralleled the divisions of the portal vein up to the interlobular vessels and always showed regular profile and spindle shaped endothelial nuclear imprints. The PBP and the PPP took their origin from both the HA and from short collateral vessels of larger arteries[13,39,42].

In larger portal spaces, the PBP is an anastomotic network between short collateral arterioles from arising from the same arterial branch. The network presents two layers only next to the hilum, being generally mono-layered. In smaller portal spaces the PBP is progressively reduced up to one single capillary[13,39].Capillaries are recognized by the poorly defined endothelial imprints and the irregular profile of the cast[37,43]. Sometimes, capillaries both from the PBP, the PPP as well as single capillaries, were more dilated than normal but always exhibited a smaller diameter than adjacent sinusoids[13,39]. The PPP can be recognized as a true net only in very large portal spaces. However, in other regions the PPP is represented by interstitial, isolated capillaries. In smaller spaces it is not possible to discern periportal from peribiliary capillaries[13,39].

The extrahepatic biliary tree shows a dense vascular network arranged around the circumference of the common bile duct[15]. Arterial and venous trunks are very easily distinguished by observing their different endothelial imprints on the cast surface. Two main layers

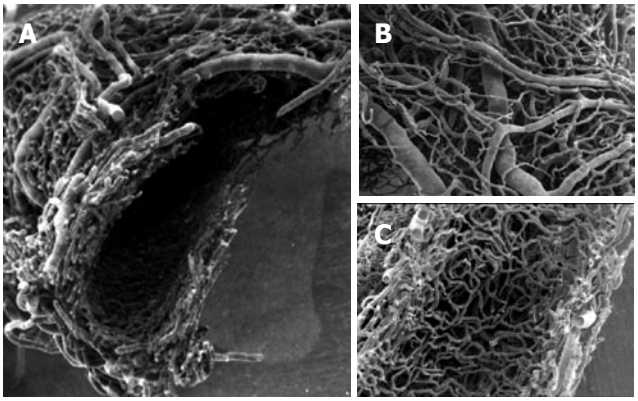


Figure 1 SEMvcc of normal rat liver (orig. magn., 20×). A dense vascular network is arranged around the circumference of the common bile duct. **A**: transverse section of Extrahepatic peribiliary plexus. Observe the arterial and venous vessels on the outer layer (**B**) and a dense capillary network in the inner layer of the plexus (**C**).

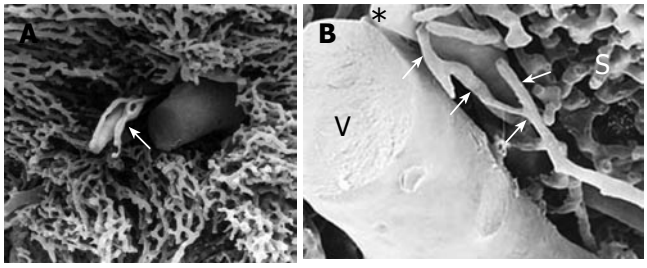


Figure 2 SEMvcc of normal rat liver (orig. magn., **A**: 40×; **B**: 110×). (**A**) small portal tract: PBP is characterized by a single layer of capillaries. (**B**) * = terminal hepatic artery, V = vena porta, arrows = peribiliary plexus, S = sinusoids.

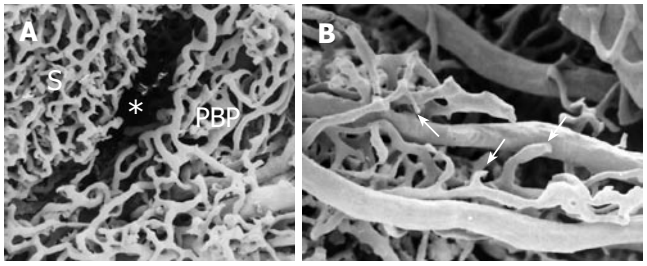


Figure 3 SEMvcc of BDL rat liver (orig. magn., **A**: 40×; **B**: 60×). (**A**) proliferated PBP run at the periphery of the lobule separated from the sinusoidal network (S) by an empty space (*) which corresponds to proliferating connective tissue. (**B**) not completely developed PBP with typical neovascular organization with dead end vessels (arrows).

were recognized in the cast. Closely meshed arterial and venous vessels formed the outer layer of the plexus and a dense capillary network in the inner layer (Figure 1). The arterial network (30-40 μm) ramifies into precapillaries. At the origin of some of precapillary vessels, an indentation produced by perivascular cells or the rearrangement of smooth muscle fibers can be observed[40,44]. The capillaries that originate from precapillary arterioles form a network plane organized in the same plane of the arterial and venous networks and in a deeper, denser and stratified capillary network. This capillary plane, if observed in its internal surface, exhibited well-delineated pits where the capillaries are arranged around a vascular space that, at LM observation, correspond to small acinar glands surrounded by thin capillaries. Large venous vessels can be observed at the same plane of the arterioles; these drained blood arising from both the superficial and inner capillary networks[15].

The extrahepatic PBP progressively continued with the intrahepatic PBP. It was composed of afferent arterial vessels and capillaries and was observed more easily in large portal tracts. In small portal tracts, the PBP was characterized by a single layer of capillaries that typically defined the periphery of the hepatic lobule (Figure 2).

PBP supports the bile epithelial function: the extension of the extrahepatic peribiliary tree underlies the ability of the bile duct to absorb water and electrolytes during the passage of bile through this collecting system[45]. Furthermore, the absence of the gallbladder in the rat emphasizes the role of EPBP in water reabsorption during the interdigestive phase[15]. Finally, the connection between the inner capillary venular plexus, portal venules and sinusoids, permits the transport of substances directly to the liver parenchyma where they can exert their actions. The reduction of complexity of the PBP inside the liver is strictly correlated with the progressive decrease of the diameter of the small bile duct, hence in the smaller portal tract the PBP can be reduced to only few capillaries[15].

Cholangiocyte proliferation started after BDL at the edge of the portal tract. During the first week from BDL the hepatic microcirculation did not show any alterations with respect to the normal liver. But after two weeks from BDL SEMvcc showed extention of the PBP from the portal tract. The proliferated PBP presented a three-dimensional net that did not arrange around a single lumen but delimited different troughs with a diameter of 20-30 μm. An empty space separated proliferated PBP and portal tract vessels from sinusoids. The sinusoids showed a normal organization with the typical structure of a classic lobule, in which sinusoids run evidently towards the central vein[9,13,15,24-26].

Three to four weeks after common BDL, a typical well-developed peribiliary microvascular plexus, originating from arterioles derived from hepatic arterial branches, is observed. The plexus runs at the periphery of the lobule and appears hypertrophic, but otherwise normal in its arrangement. The plexus is composed of many layers and shows an intimate meshed network, characterized by round loops, resembling the organization of the inner vascular layer of the extrahepatic peribiliary plexus (Figure 3A). Between the PBP and the sinusoidal network there is an empty space, which corresponds to proliferating connective tissue, digested during the casting procedure (Figure 3A). Infrequent vascular communications between the PBP and sinusoids are also visible. The efferent vessels that arise from the confluence of the capillaries form a small vein that tends to drain into the interlobular vein. In some areas of the liver the PBP does not seem to develop completely. In these areas there is a typical neoangiogenetic organization with interrupted vascular loops and dead-end vessels (Figure 3B). These structures may represent an attempt to increase metabolic exchange within the ductular lumen and PBP[45,46]. In contrast to the profound

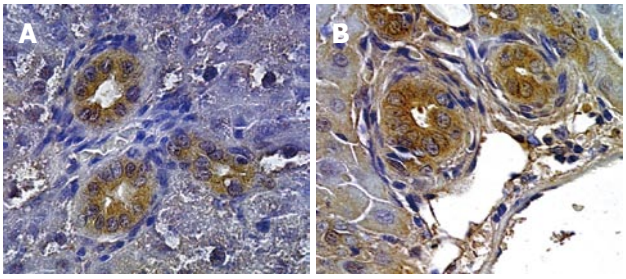


Figure 4 Immunohistochemistry for VEGF-A (**A**) and VEGF-C (**B**). Proliferating bile ducts in BDL rat show an intense positivity for both VEGF-A and VEGF-C (Orig. magn., 20×).

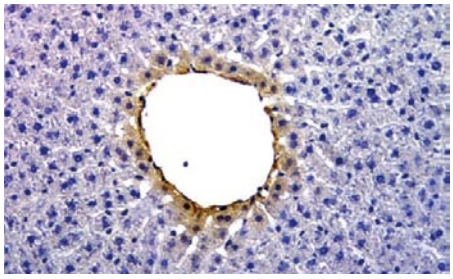


Figure 5 Immunohistochemistry for VEGF-A. Normal rat liver. The hepatocyte of pericentral zone shows positivity for VEGF-A (Orig. magn., 20×).

modification of the PBP, the sinusoidal organization remains quite normal even in BDL rats.

After BDL, the intrahepatic biliary epithelium undergoes cholangiocyte proliferation[48,50], which leads to bile duct mass expansion, followed by an adaptive proliferation of the PBP[9]. Proliferating bile ducts are characterized by enhanced cholangiocyte secretory and proliferative activities[47]. Cholangiocyte proliferation is modulated by a complex system of growth factors, neuropeptides and hormones[51-54]. Most of this information has been achieved in the BDL-rat model, which is characterized by cholangiocyte proliferation[55-57] followed by an adaptive proliferation of the PBP[9] that occurs after that of the intrahepatic biliary epithelium. Therefore, the adaptive proliferation of the PBP (and its circulating factors including VEGF) seems to be adequate for the increased mass of the bile ductal system.

VEGF REGULATION

Considering the enormous extension of the PBP during BDL, it is interesting to evaluate the possible role played by one of the most potent and well known angiogenic factors, the VEGF. VEGF is a member of a family of related growth factors that includes VEGF-A, -B, -C, -D, and -E, and placenta growth factor[58-61]. VEGF's role in vascular proliferation associated with tumour growth or wound healing has been widely documented in different organs[62]. The expression of VEGF and its receptors is not restricted to vascular endothelial cells, since their expression has been detected in vascular smooth muscle cells, osteoblasts, regenerating myotubes and hematopoietic stem cells[59-61,63]. Moreover, VEGF has also been secreted in a large number of normal epithelial cells, such as keratinocytes, goblet cells in nasal polyps, pulmonary cells, prostate cells, ductal cells derived from normal pancreas and also in normal hepatocytes[66-68]. Because cholangiocyte proliferation, as described above, is regulated by growth hormones, we investigated the expression of VEGF and its receptors in normal and proliferating biliary epithelia.

After immunohistochemistry of liver sections from normal and BDL rats, we found that intrahepatic cholangiocytes from both normal and BDL rats express VEGFR-2 and VEGFR-3 but not VEGFR-1[38]. This data was confirmed by immunoblot analysis in a purified cholangiocyte cell culture. Moreover, we found that intrahepatic cholangiocytes from normal and BDL rats express the protein for VEGF-A and VEGF-C[38]. Immunohistochemistry shows that the expression of VEGF-A and VEGF-C was higher in bile ducts from BDL rats (Figure 4) compared to bile ducts from normal rats[38]. In addition, we found VEGF in the supernatant of primary cultures of normal cholangiocytes, indicating that the intrahepatic cholangiocytes secrete VEGF. Following BDL, the amount of VEGF secreted by cholangiocytes into the supernatant significantly increased compared to the amount of VEGF secreted by normal cholangiocytes. In normal rat liver, pericentral hepatocytes show positivity for VEGF-A (Figure 5).

In order to confirm that VEGF plays an important role in the regulation of cholangiocyte proliferation, we also tested the effect of the administration of an anti-VEGF-A or an anti-VEGFC antibody to BDL rats. The administration of anti-VEGF to BDL rats induced an increased number of apoptotic cholangiocytes compared with BDL and a decrease in the number of PCNA and CK-19 positive cholangiocytes. In order to demonstrate that the proliferation of the PBP following BDL only occurs after cholangiocyte proliferation, we measured the number of PCNA-positive vascular endothelial cells and cholangiocytes in liver sections from rats with BDL for 1 or 2 wk. After BDL for 1 wk, PCNA was expressed only by proliferating cholangiocytes (and in rare hepatocytes), whereas endothelial cells were negative. In contrast, after BDL for 2 wk, both cholangiocytes and endothelial cells became positive for PCNA. This was confirmed by the immunohistochemical expression of Factor VIII-related antigen (a marker of endothelial cells)[69] in liver sections from normal, 1- and 2-wk BDL rats. The number of Factor VIII- related antigen positive cells increase, in comparison with normal rats, only after 2-wk BDL but it was unchanged after 1-wk BDL, thus confirming that the proliferation of PBP occurs after that of the biliary epithelium.

In addition, to better define the role of VEGF-A in the regulation of cholangiocyte and PBP proliferation during BDL we performed in: (1) normal rats and normal rats + hepatic artery ligation (HAL); (2) 1 wk BDL[4,5] rats; and (3) rats that (immediately after BDL or BDI + HAL) were treated by IP implanted with BSA or r-VEGF-A for 1 wk[70-73]. With the aim of evaluating PBP organization and the cholangiocyte VEGF protein expression, SEMvcc were performed. The peribiliary plexus, observed in BDL rats[9], was not demonstrated in BDL + HAL rats by the

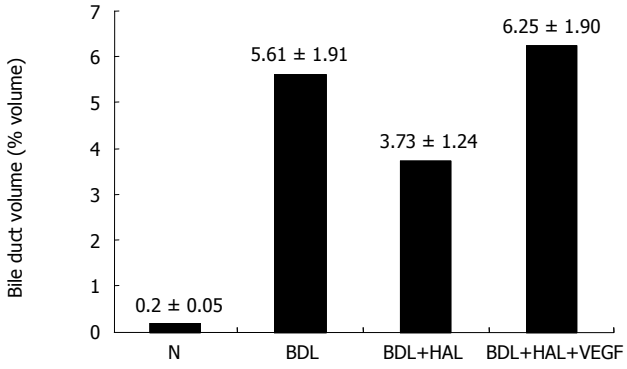


Figure 6 Effect of hepatic artery ligation and chronic administration of VEGF on bile duct volume.Bile Duct volume is measured as a volume fraction of the entire liver tissue specimen. HAL induced in BDL rats a disappearance of PBP and an impaired cholangiocyte proliferation. The effects of HAL on PBP and cholangiocyte functions were prevented by r-VEGF by maintaining the integrity of the peribiliary plexus and cholangiocyte proliferation.

SEMvcc technique. We did not observe PBP in BDL + HAL rats because the PBP is nourished by the HA. In normal rats, PBP was observed more easily in large portal tracts[9]. In small portal tracts, the PBP was characterized by single layer of capillaries or even by a single capillary; in BDL + HAL we do not observed PBP. Administration of r-VEGF-A to BDL + HAL rats prevented the HAL-induced microvascular modification (absence of PBP). The microvascular pattern observed after administration of r-VEGF-A to BDL + HAL rats demonstrated the presence of a PBP with similar characteristics previously described in BDL rats[9,70].

Immunohistochemistry in BDL liver sections shows that bile ducts express VEGF-A, VEGFR-2 and VEGFR-3. HAL induced a decrease in the number of cholangiocytes positive for VEGF-A and VEGFR-2 and VEGFR-3 receptors compared to liver sections from 1 wk BDL rats. Following the administration of r-VEGF-A to BDL + HAL rats, the expression of VEGF-A and VEGFR-2 and VEGFR-3 was similar or higher than that of BDL rats. VEGFR-1 was not expressed by cholangiocytes. These indicate that VEGF, predominantly by an autocrine mechanism, plays an important role in modulating cholangiocyte proliferation[70]. Furthermore, if we drastically reduced the HA blood flow in BDL rats by the HAL, we induced the absence of PBP, reduction of both VEGF and VEGF receptors at the cholangiocyte level and a reduction of bile duct mass with no induced effect in the liver of normal rats (Figure 6). Evidently, when the intrahepatic bile duct mass is expanded, as occurs after BDL[47], blood supply through the hepatic artery becomes fundamental in sustaining the enhanced nutritional and functional demands of proliferating intrahepatic biliary epithelium[9]. VEGF has been demonstrated to participate in a complex that sustains cholangiocyte proliferation after BDL[50]; therefore, concerning the effect of HAL on bile duct mass, the fall of synthesis and release of VEGF by cholangiocytes after HAL certainly has a role in compromising cell proliferation.

In conclusion, recent data highlights the role of arterial blood supply of biliary tree in conditions of cholangiocyte proliferation, such occurs during chronic cholestasis. On the other hand, the role played by VEGF as a tool of cross-talk between cholangiocytes and PBP endothelial cells suggests that manipulation of VEGF release and function could represent a therapeutic strategy for human pathological conditions characterized by damage of hepatic artery or the biliary tree.

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Bile acid interactions with cholangiocytes

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Abstract

Cholangiocytes are exposed to high concentrations of bile acids at their apical membrane. A selective transporter for bile acids, the Apical Sodium Bile Acid Cotransporter (ASBT) (also referred to as Ibat; gene name Slc10a2) is localized on the cholangiocyte apical membrane. On the basolateral membrane, four transport systems have been identified (*t*-ASBT, multidrug resistance (MDR)3, an unidentified anion exchanger system and organic solute transporter (Ost) heteromeric transporter, Ostα-Ostβ. Together, these transporters unidirectionally move bile acids from ductal bile to the circulation. Bile acids absorbed by cholangiocytes recycle via the peribiliary plexus back to hepatocytes for re-secretion into bile. This recycling of bile acids between hepatocytes and cholangiocytes is referred to as the cholehepatic shunt pathway. Recent studies suggest that the cholehepatic shunt pathway may contribute in overall hepatobiliary transport of bile acids and to the adaptation to chronic cholestasis due to extrahepatic obstruction. ASBT is acutely regulated by an adenosine 3', 5'-monophosphate (cAMP)-dependent translocation to the apical membrane and by phosphorylation-dependent ubiquitination and proteasome degradation. ASBT is chronically regulated by changes in gene expression in response to biliary bile acid concentration and inflammatory cytokines. Another potential function of cholangiocyte ASBT is to allow cholangiocytes to sample biliary bile acids in order to activate intracellular signaling pathways. Bile acids trigger changes in intracellular calcium, protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), mitogen-activated protein (MAP) kinase and extracellular signal-regulated protein kinase (ERK) intracellular signals. Bile acids significantly alter cholangiocyte secretion, proliferation and survival. Different bile acids have differential effects on cholangiocyte intracellular signals, and in some instances trigger opposing effects on cholangiocyte secretion, proliferation and survival. Based upon these concepts and observations, the cholangiocyte has been proposed to be the principle target cell for bile acids in the liver.

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Key words: Cholangiocytes; Bile acid; Liver

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INTRODUCTION

In earlier studies of hepatobiliary physiology, it was generally concluded that biliary components do not appreciably interact with bile ducts and after canalicular secretion, bile acids and lipids pass through the biliary system as if bile ducts were inactive conduits[1]. Twenty-five years ago, the cholehepatic shunt pathway was proposed to explain the hypercholeretic nature of certain bile acids[2,3]. This hypothesis suggested that bile acids, in a protonated, uncharged form, undergo passive biliary absorption, followed by transfer of bile acids back to hepatocytes for re-secretion into bile (Figure 1)[2]. Later, other studies suggested that in the presence of complete bile duct obstruction, canalicular secretion of bile acids persists and bile acids may pass back through cholangiocytes to the circulation instead of the entering the intestine[4]. Subsequent to the discovery of the expression of a bile acid transporter on the apical membrane of cholangiocytes (apical sodium-dependent bile acid transporter or ASBT)[5-8], there has been renewed interest in the potential of cholehepatic shunting of bile acids. Cholangiocyte ASBT adapts to chronic cholestasis induced by bile duct obstruction by upregulation of cholangiocyte

transport capacity potentially leading to augmentation of cholehepatic shunting of bile acids and protection from cholestatic liver injury due to hepatocellular retention of bile acids[9].

Bile acids also interact with cholangiocytes leading to alteration of cholangiocyte secretion, proliferation, apoptosis and differentiation[7,8,10-12]. In both hepatocytes and stellate cells, bile acids when present intracellularly in low concentrations (e.g. less than 10 μmol/L) function as intracellular signals triggering wide variety of protein kinases, changes in intracellular Ca^{2+} and phosphorylation of proteins. Since bile acids do not appreciably enter cells in the absence of a membrane transporter, the expression of bile acid transporter is required for bile acid signaling, and the degree of bile acid transporter expression may determine the sensitivity of cells to bile acid signaling. Thus the bile acid transporter functions in some respects like a membrane receptor, determining both selective and sensitivity of the cell reactions to bile acid agonists. Bile acids, alter Ca^{2+}, cAMP, PKC and PI3K intercellular signaling systems in cholangiocytes. ASBT function was shown to be required for signaling and the level of ASBT expression correlated with cholangiocyte sensitivity to bile acids[7,8,10,13,14]. As a manifestation of the signaling properties of bile acids in cholangiocytes, studies show that bile acids may directly stimulate cholangiocyte proliferation and secretion[11,12] and thus accumulating bile acids due to chronic cholestasis may promote the ductal hyperplasia that occurs in chronic cholestatic liver disease. Changes in biliary bile acid composition or concentration may also modulate cholangiocyte survival[15,16]. Increasing biliary bile acid concentration, by bile acid feeding, reduces cholangiocyte apoptosis induced by CCl_4 or vagotomy in rats[15,16]. As another potential signaling function, bile acids may alter cholangiocyte differentiation, since small bile ducts following chronic exposure to bile acids begin expressing proteins and functions normally only present in large intrahepatic bile ducts[7]. Therapeutic bile acids (ursodeoxycholate), perhaps surprisingly, have opposing effects on cholangiocyte function compared to endogenous bile acids. Ursodeoxycholate appears to reduce cholangiocyte proliferation and reduce bile mass in animal models of bile duct hyperplasia[8]. The implications of these findings are discussed later.

The purpose of this review is familiarizing the reader in the role of bile acids in cholangiocyte biology and pathophysiology. Recent studies unexpectedly show that cholangiocytes transport bile acids and adapt to changes in biliary bile acids. Considering that one of the fundamental events in cholestasis is the retention in the liver of components that are normally secreted in bile, alternative pathways for elimination of biliary components would be an important adaptation of the liver to cholestasis. The review will outline mechanisms for bile acid transport in cholangiocytes, the role of bile acid regulation of cholangiocyte proliferation, secretion and apoptosis in animal models and human diseases. First the mechanisms for bile acid uptake at the cholangiocyte apical membrane will be reviewed. Second, basolateral bile acid efflux mechanisms are outlined. Third, the current evidence for cholehepatic shunting of bile acids is summarized.

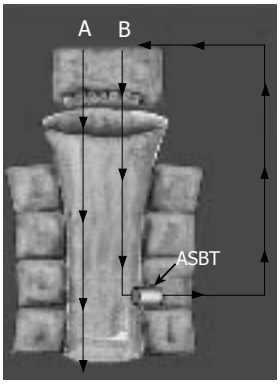


Figure 1 Classic cholehepatic shunt pathway (B) compared to direct biliary bile acid secretion (A). Bile acids that are poor substrates for coenzyme A (CoA) synthetase and inefficiently conjugated by hepatocytes are passively absorbed by biliary epithelium. After canalicular secretion, the unconjugated bile acid is absorbed by cholangiocytes because of the high lipophilicity of the protonated acid. The absorbed bile acid is returned to the hepatocyte mass *via* the periductular plexus. The osmotic effect of multiple passages of the unconjugated bile acid anions into canalicular bile results in hypercholeresis. The presence of ASBT on the apical membrane of cholangiocytes provides a potential mechanism for absorption of conjugated bile acids that then follows the same shunt pathway as described above.

Next the mechanisms responsible for acute and chronic regulation of ASBT activity in cholangiocytes are reviewed. Finally, the concept of intracellular bile acid acting as signaling molecules in cholangiocytes will be developed and the evidence for bile acid regulation of cholangiocyte secretion, proliferation and survival will be summarized.

CHOLANGIOCYTE BILE ACID UPTAKE

Although earlier studies have suggested bile acid uptake mechanisms were present in cholangiocytes, the identification of ASBT by Lazaridis *et al*[17] and Alpini *et al*[5] brought to the forefront the interest in bile acid transport in the biliary system. ASBT had previously been identified in ileum and kidney tubules[18] and ASBT has been proposed as the major transporter involved in the reclamation of bile acids in the intestine and in the nephron, respectively[18]. Studies by Alpini *et al*[5] showed the presence of gene expression for both the ASBT and ileal bile acid binding protein (IBABP) in cholangiocytes. Immunofluorescence studies showed that ABAT protein is expressed on the apical membrane of isolated cholangiocytes and isolated bile duct units (IBDU)[5]. Our studies showed the majority of [^{3}H]-taurocholate uptake is Na^+-dependent with a K_m of 43 μmol/L and a V_{max} of 190 pmol/min[5]. These values were lower compared to measurements by Lazaridis *et al*[17], however studies by Alpini *et al*[5] were performed in freshly isolated cholangiocytes (which may be a more physiological model) whereas those by Lazaridis were done in a rat cholangiocyte cell line. In addition, the kinetics for taurocholate uptake in freshly isolated cholangiocytes has a similar K_m and V_{max} as reported by other investigators for ASBT-mediated uptake in the ileum[19,20]. The K_m for a transporter is generally similar to the physiologic concentration of the transported substrate. The markedly lower K_m for ASBT in cholangiocytes compared to biliary bile acid concentration may be due to the effect of unstirred layer adjacent bile duct lumen membrane that would reduce the effective bile acid concentration immediately adjacent to the cholangiocyte apical membrane[21]. Lazaridis *et al*[17] demonstrated vectorial transport of bile acids from apical to basolateral direction and an absence of transport in the basolateral to apical direction in a normal rat cholangiocyte

cell line in a polarized culture system. No additional bile acid uptake proteins have as of yet been identified in the cholangiocyte apical membrane.

The natural substrate specificity of ASBT is narrow and restricted to unconjugated bile acids as well as their glycine- and taurine-conjugates[22]. ASBT appears to play a major role in the enterohepatic circulation of bile acids since dysfunctional mutations in the mouse or human ASBT genes cause profound bile acid malabsorption in humans[23]. In the renal tubule ASBT acts as a salvage mechanism to prevent urinary excretion of bile acids that undergo glomerular filtration. The role of ASBT in bile ducts is not as obvious as in the intestine and kidney. We have proposed that ASBT functions in a cholehepatic shunt where bile acids are absorbed in the biliary tract, secreted into the periductular capillary plexus, and carried directly back to the hepatocyte for secretion, thereby promoting bile flow. Alternatively, ASBT may function to sense bile acid concentration in bile, a mechanism that is dependent on bile acid uptake by ASBT and bile acid-dependent activation of intracellular signaling systems in cholangiocytes. The principles of cholehepatic shunting and bile acid signaling are discussed in a later section.

Intracellular bile acid movement in cholangiocytes

In hepatocytes, cytosolic binding proteins have been shown to sequester bile acids in a bound state that may prevent the cytotoxicity of free intracellular bile acids[24,25]. The presence of high affinity binding sites would significantly reduce the rate of transcellular transport of bile acids. Indeed, previous studies have shown that the transcellular transport comprises the greatest proportion of time in the overall transcellular transport of conjugated bile acids in hepatocytes[26].

The ileal bile acid binding protein (IBABP) is expressed in cholangiocytes[5]. Very little is known as to whether it functions to prevent intracellular toxicity, modulates transcellular transport or it changes in expression in response to increased bile acid flux in cholangiocytes or in the presence of cholestasis. Recent studies show expression of IBABP in the ileum is regulated by bile acid concentrations through the effects of farnesoid X receptor[27].

Bile acid efflux in cholangiocytes

Since Lazaridis *et al*[17] studies have shown that bile acid transport in cholangiocytes is vectorial (e.g. apical-to-basolateral), mechanisms are likely present in the basolateral membrane that facilitates the efflux of bile acids into peribiliary plexus circulation. Four mechanisms have been identified in previous studies that together account for bile acid efflux in cholangiocytes. The first mechanism was identified employing bile duct fragments. The studies showed that fluorescent bile acid analogs can be taken up across the cholangiocyte basolateral membrane[28]. The uptake process involved an anion exchanger mechanism that was identified by inhibition of bile acid uptake in the absence of Cl^- or HCO_3^- or the presence of 4, 4'-diisothiocyanostilbene-2, 2'-disulfonic acid[28]. Although the authors studied uptake across the basolateral membrane in their model, they proposed that their studies reflected physiologically an anion exchanger that effluxes bile acids out of cholangiocytes subsequent to apical uptake. The protein responsible for this anion exchanger was not identified.

The second mechanism for bile acid efflux was identified by Lazaridis *et al*[6]as an exon-2 skipped, alternatively spliced form of ASBT, designated *t*-ASBT. Alternative splicing causes a frameshift that produces a 154-aa protein. *T*-ASBT is expressed in rat cholangiocytes, ileum, and kidney and is localized to the basolateral domain of cholangiocytes[6]. Transport studies in *Xenopus* oocytes revealed that *t*-ASBT functions as a bile acid efflux protein. Compared to ASBT, alternative splicing changes the cellular targeting of ASBT provides a mechanism for rat cholangiocytes to efflux bile acids at the basolateral membrane[6]. The regulation of *t*-ASBT expression in response to biliary or circulatory bile acid concentrations or in the presence of cholestasis has not been determined. The regional distribution of *t*-ASBT in large and small ducts is not known. The third mechanism for bile acid efflux in cholangiocytes is MDR3. Previous studies have shown that MDR3 is expressed on the basolateral membrane of cholangiocytes and MDR3 is upregulated in chronic cholestasis associated with type 3 progressive familial intrahepatic cholestasis[29]. It has been proposed that up-regulation of MDR3 may promote cholehepatic shunting in chronic cholestasis, thus preventing the toxic effects of accumulating bile acids in cholangiocytes[29]. Similar upregulation of MDR3 in hepatocyte basolateral membranes has been proposed to pump bile acids out of hepatocytes with canalicular cholestasis[30] and MDR3 is upregulated in patients with Dubin-Johnson syndrome[31]. There is no direct evidence that shows MDR3 effluxes bile acids in cholangiocytes or provides a mechanism for prevention of accumulation of bile acids in cholangiocytes during cholestasis.

Recently the Ostα-Ostβ heteromeric transporter was initially identified in the liver, ileum, and kidney[32]. In contrast to all other organic anion transporters identified to date, transport activity requires the coexpression of both Ostα and Ostβ proteins. Substrates for this transporter include the bile acid taurocholate, other steroids (estrone 3-sulfate and digoxin), and prostaglandin E2[33]. Ostα and Ostβ mRNA expression along the mouse gastrointestinal tract mirrors that of ASBT, and both Ostα and Ostβ proteins are localized to the basolateral surface of ileal, renal and bile duct cells[32]. Studies of bile acid transport in Ostα and Ostβ expressing *Xenopus laevis* oocytes showed bile acid efflux and trans-stimulation, indicating that transport occurs by facilitated diffusion. The selective localization of Ostα and Ostβ to the basolateral plasma membrane of epithelial cells responsible for bile acid and sterol reabsorption, the substrate selectivity of the transporter, suggest that heteromeric Ostα and Ostβ is an important basolateral bile acid transporter in biliary, ileal and renal epithelial cells.

Cholehepatic shunting of bile acids

Hoffman proposed bile acids may cycle between cho-

langiocytes and hepatocytes through a cholehepatic shunt pathway[3]. Unconjugated bile acids[34] or non-charged bile acids (norursodeoxycholate)[34] were observed to induce a greater degree of bile flow per bile acid molecule excreted in bile. To account for this hypercholeretic effect, it was proposed that unconjugated bile acids may be passively absorbed by bile ducts; enter the peribiliary plexus adjacent to intrahepatic bile ducts, then forwarded to the hepatic sinusoids to be returned to cholangiocytes by hepatocyte secretion[3]. Typically, cholehepatic shunting initiated by passive absorption of non-ionized bile salt results in the generation of bicarbonate molecules in bile, which then increases biliary bicarbonate excretion. Other criteria for cholehepatic shunting besides hypercholeresis and alkalinization of bile have been identified. With the hypercholeresis, the biliary transit time for the unconjugated or non charged bile acids was greater than expected which would be predicted by longer retention time in the liver due to more than one passage through the hepatobiliary axis[34]. Back perfusion of the isolated perfused liver (infusion into the hepatic vein), a route where the blood from the peribiliary plexus does not appreciably enter the hepatic sinusoids, reduced the hypercholeretic effect of ursodeoxycholic acid[35]. Increased number of bile ducts in animal models of cirrhosis was associated with increased hypercholeresis due to ursodeoxycholic acid infusion, an observation that was attributed to increased cholehepatic shunting due to increase bile duct mass[36]. Similarly, bile duct proliferation in Mdr2(-/-) mice is associated with a disproportionably high bile flow in response to tauroursocholate acid infusion, a finding that was interpreted as due to enhanced cholehepatic shunting of bile salts due to increased number of bile ducts. The magnitude of absorption of bile acids under physiologic or pathophysiologic conditions in man is not known.

Identification of apical and basolateral bile acid transport proteins in cholangiocytes, points to the possibility that the cholehepatic shunt pathway functions in bile secretion and may adapt in response to cholestasis. From a functional point of view, the pathway provides a mechanism to enhance bile acid-dependent bile flow and biliary lipid excretion. With multiple passages of bile acids through the canalicular membrane (as a result of recycling through cholangiocytes), the cholehepatic shunt pathway has the potential to increase the efficiency of bile acid-induced biliary lipid excretion and bile acid-dependent bile flow[3]. From the pathophysiologic point of view, the pathway provides an alternative route for continuation of hepato-cholangiocyte flux of bile acids despite the presence of complete bile duct obstruction[37]. The latter may well be an important pathophysiologic response of the liver to bile duct obstruction.

In support of ASBT initiating cholehepatic shunting, our studies[38] have shown that following the administration of secretin to bile duct ligated rats, there is acute upregulation of ASBT in cholangiocytes (as described in the next section). With secretin stimulation of ASBT activity in cholangiocytes, there is a marked increase in taurocholate-induced choleresis (12 ± 2 μL per μmol bile acid excreted with basal cholangiocyte ASBT activity compared to 38 ± 6 μL per μmol bile acid excreted during high cholangiocyte ASBT activity). Similarly, with experimental augmentation of cholangiocyte ASBT activity, taurocholate induces a much greater increase in biliary phospholipid (1.5-fold increase in μmol phospholipid per μmol bile acid excreted compared to basal) and cholesterol secretion (2-fold increase in μmol cholesterol per μmol bile acid excreted compared to basal). Finally, following the administration of secretion, the taurocholate transit time is increased by 7 min. The taurocholate-induced hypercholeresis, increased biliary lipid excretion and increased taurocholate transit time are consistent with enhanced taurocholate cholehepatic shunting due to up regulation of ASBT by secretin. Additional studies will be needed to establish the degree of cholehepatic shunting of conjugated bile acids in normal rats.

OVERVIEW OF THE REGULATION OF ASBT EXPRESSION IN CHOLANGIOCYTES

Alteration of bile acid transporter activity may occur physiologically in response to local bile acid concentrations to fine tune bile acid transport capacity. For instance, ASBT has been shown by some studies to be upregulated in the ileum with increased intestinal bile concentration[39], so as to provide increased intestinal bile acid transport activity in response to increased intestinal bile acid load. Alternatively, bile acid transporter expression may be chronically modified in pathologic conditions to prevent intracellular accumulation of toxic bile acids due to altered bile acid metabolism or retention[40]. For instance, in chronic cholestasis due to bile duct obstruction, there is downregulation of hepatocyte sinusoid transporters and upregulation of hepatocyte canalicular transporters that provides the combined effect to reduce hepatocyte intracellular retention of bile acids that occurs with cholestasis[41]. Regulation of bile acid transporters may also occur regionally within anatomical confines of an organ. For instance, ASBT expression is present exclusively in the distal ileum (and not proximal small bowel)[42], restricted to the mature enterocytes lining the villus, with little or no detectable expression in the small intestinal crypt. Sinusoidal transporters are present to a greater degree in the periportal region compared to the pericentral region of the hepatic lobule[41]. Temporally, regulation of bile acid transporters has been shown to occur gradually through changes in gene expression[39,40,43,44], or acutely by changes in cell membrane transporter content by transporter translocation[45,46] or by changes in the rate of protein degradation.

Acute regulation of ASBT in cholangiocytes

Previous studies have shown that ASBT transport activity acutely increases in cholangiocytes and ileal epithelial cells by a cAMP-dependent mechanism[38,47]. In cholangiocytes, increased cAMP induced by secretin doubles Na^+-dependent bile acid uptake in isolated cholangiocytes[38] and in perfused bile duct fragments[48]. This effect is likely due to protein translocation, since pretreatment of cholangiocytes with the microtubule inhibitor colchicine prevents the cAMP-induced increase of Na^+-dependent

bile acid transport[38]. The effects of secretin on protein translocation of ASBT to the apical membrane of cholangiocytes were studied employing isolated apical membranes from cholangiocytes[38]. These studies showed that cAMP increases apical membrane ASBT only in the absence of colchicine. A model for cAMP-dependent recycling of ASBT in the cholangiocyte apical membrane is shown in Figure 2. The model shows that cAMP increases apical ASBT membrane content, but also suggests that ASBT recycles back to latent intracellular stores once the secretin/cAMP stimulus has abated. This mechanism for acute induction of ABAT activity in cholangiocytes has been proposed to provide an accentuation of cholehepatic bile acid shunting in the postprandial period, thus accentuating bile flow and biliary lipid secretion (see cholehepatic shunting section above)[38].

Chronic regulation of ASBT expression in cholangiocytes

ASBT expression in cholangiocytes changes chronically in response to biliary bile acid concentrations, the presence of cholestasis[7-9,15] or inflammation. With increase in biliary bile acid concentration, due to feeding taurocholate to rats, there is an increase in total liver ASBT[7]. In this model, the increased ASBT is due to both increased number of cholangiocytes in the liver and the maintenance of ASBT expression per cell. In bile duct ligated rats depleted of biliary bile acids for 12 h by external biliary drainage, there is a marked decrease in cholangiocyte ASBT gene and protein expression and transport activity[49]. ASBT gene and protein expression and transport activity can be restored in bile-depleted rats by infusion of taurocholate to maintain biliary bile acid concentration[49]. These studies employing bile acid feeding and bile acid depletion show that ASBT expression in cholangiocytes is chronically regulated in a direct proportion to biliary bile acid concentration. In contrast to taurocholate feeding which increases cholangiocyte ASBT expression, feeding ursodeoxycholic acid to bile duct ligated rats markedly reduces cholangiocyte ASBT gene and protein expression and taurocholate transport activity[8]. Although the mechanism for differential effects of different bile acids on ABAT expression in cholangiocytes has not been defined; they are consistent with our studies showing differential effects of different bile acids on cholangiocyte secretion and proliferation (see below).

With chronic cholestasis due to bile duct ligation, intrahepatic bile ducts markedly increase in number (approximately 10 fold increase after 1 wk). In this model, ASBT expression per cholangiocyte is maintained[9], so that overall there is an effective increase in biliary bile acid absorptive capacity in BDL rats. We propose that the increased ASBT in BDL rats provides an alternative excretory pathway in the presence of biliary obstruction so as to prevent the bile acid stasis in the liver and the subsequent accumulation of toxic bile acids in hepatocytes[9].

Recently it was demonstrated that bile acids modulate ASBT expression through activation of the peroxisome proliferator-activated receptor alpha (PPARalpha)[18] and activator protein 1 (AP-1) element regulates the transcription of the rat ASBT gene[44].

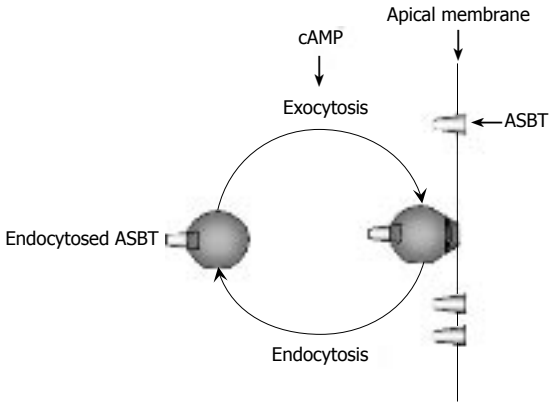


Figure 2 Membrane recycling of ASBT. Increased cAMP enhances bile acid uptake in cholangiocytes. Under basal conditions, intracellular ASBT resides in an inactive position within the cytoplasm of cholangiocytes as well as on the apical membrane. Increased cAMP results in translocation of ASBT to the apical membrane where it inserts by exocytosis. Once on the apical membrane, ASBT becomes active and mediates absorption of bile acids from bile. Membrane recycling of ASBT is completed by removal of ASBT from the apical membrane by endocytosis.

Previous studies have shown that the stress induced alteration of ASBT genetic expression due to inflammation and bile acids is a consequence of trans-activation of the ASBT promoter by c-Jun/c-Fos and liver receptor homologue-1, respectively[10,47] and that hepatocyte nuclear factor-1α is critical for basal expression of ASBT.

Recently, the inflammatory cytokine IL-1β has been shown to rapidly down-regulate ASBT in the terminal ileum[50]. Dysregulation of the ASBT adaptation to cholestasis (due to increased expression of IL-1β) could blunt the compensatory up-regulation of ASBT in response to cholestasis and promote bile acid-induced liver damage. Recent data demonstrates that the ubiquitin-proteasome degradation system affects the activity of some membrane transporters[51,52]. The system is responsible for the disposal of many of the short-lived proteins in eukaryotic cells. The ubiquitin-proteasome pathway targets proteins for degradation *via* covalent tagging of the substrate protein with a polyubiquitin chain[53]. This degradation pathway is implicated in the regulation of many short-lived proteins involved in essential cellular functions, including cell cycle control, transcription regulation, signal transduction, and protein translocation. We speculated that the initial ASBT down-regulation due to ileal inflammation or due to IL-1β *in vitro* is caused by enhanced ASBT disposal by the ubiquitin-proteasome pathway. Our studies showed that ASBT is an unstable protein that is rapidly degraded with a half-life of approximately 6 h. We showed that the rapid IL-1β -dependent reduction of ASBT in cholangiocytes is due to increased ASBT disposal *via* the ubiquitin-proteasome pathway. IL-1β mediated down-regulation of ASBT expression requires phosphorylation of ASBT since mutation of two ASBT phosphorylation sites reduces the rate of ASBT disposal under basal conditions and markedly reduces IL-1β -dependent ubiquitination and disposal of ASBT. These results indicate that the proteasome plays an important role in the regulation of ASBT protein level in cholangiocytes.

Regional ASBT expression in the biliary tree

Regionalization of bile duct function occurs in rat liver[5]. Large (greater than 20 μm diameter bile ducts) that are lined by large cholangiocytes contribute to hormone-induced ductal secretion whereas small (smaller than 20 μm diameter bile ducts) do not contribute to hormone-induced ductal secretion[54,55]. Studies by Alpini *et al*[5] showed that ASBT is expressed in large cholangiocytes but not small cholangiocytes. The absence of ABST expression in small intrahepatic bile ducts may lead to more efficient hepatobiliary excretion of bile acids, since bile acid uptake in small ducts, closely adjacent to the canalicular bile acid secretion process, may hinder the post canalicular assembly of polymolecular bile acid-lipid micelles and vesicles. Recently, experimental models have been developed where ASBT gene, and protein expression and transport activity have been shown to extend into small bile ducts[7]. In taurocholate fed rats, a model where biliary bile acid concentrate increases approximately two fold, Alpini *et al*[7] found *de novo* ASBT expression in small ducts. The authors suggested that with expansion of the bile acid pool and increased biliary bile acid concentration, the extension of ASBT expression into small ducts leads to enhanced cholehepatic shunting of bile acids. Whether ASBT expression in small ducts alters bile acid-lipid micelles or vesicle formation has not been determined.

BILE ACID SIGNALING IN CHOLANGIOCYTES

Overview

In a variety of cells, bile acids have been shown to function as intracellular signals and to profoundly alter cellular functions such as proliferation, differentiation, secretion, and apoptosis[56-61]. These studies have shown that cellular uptake is required for bile acids to signal cellular processes. In cells not expressing a bile acid transporter, (which normally do not respond to the presence of bile acids in the media) experimental expression of a bile acid transporter activates *de novo* bile acid signaling[62]. Once intracellular, bile acids at concentrations of less that 1 μmol/L, have been shown to alter intracellular Ca^{2+}, PKC, MEK, ERK and PI3K pathways in hepatocytes or cholangiocytes[16,59,61,63-66]. Through these downstream signals, bile acids have been shown to alter cell proliferation, secretion, apoptosis and gene expression[8,16,61,63-67]. In addition, bile acids have been shown to induce activation (by phosphorylation) of the EGF receptor in hepatocytes[64] and cholangiocytes[68]. The signaling effects of bile acids should be distinguished from the toxic effects of bile acids, where bile acid in high concentration, through changes in membrane lipids induces, in a nonspecific manner, cellular damage[69].

Bile acid signaling of cholangiocyte secretion

It had previously been observed that ursodeoxycholic acid increases biliary bicarbonate excretion into bile. Shimokura *et al*[66] found that ursodeoxycholic acid directly increases cholangiocyte secretion. They demonstrated ursodeoxycholic acid increases intracellular Ca^{2+} and increases chloride channel activity in a malignant cholangiocyte cell line[66]. The ursodeoxycholate effect on chloride channel activity was dependent on the increase in intracellular Ca^{2+}. Our studies have shown that in freshly isolated cholangiocytes, taurocholic acid or taurolithocholic acid (1-20 μmol/L) increases secretin-stimulated cAMP levels and secretin-stimulated Cl^-/HCO_3 exchanger activity[12]. These effects were dependent on taurocholate uptake by ASBT, since the stimulatory effect of taurocholate was not present in the absence of Na^+[12]. Dependence of bile acid effects on cholangiocyte secretion and ASBT transport activity, we found the K_m for ASBT in cholangiocytes to be close to the concentration where in bile acids exert their maximum response in cholangiocytes[12]. Similar to their effect *in vitro*, taurocholate or taurolithocholate feeding to normal rats for 7 d increased secretin-stimulated cAMP and Cl^-/HCO_3^- exchanger activity in cholangiocytes and resulted in an increase of secretin-stimulated ductal bile flow *in vitro*[7]. The studies show that both *in vitro* and *in vivo*, bile acids can augment secretin-stimulated ductal bile flow but the intracellular signals responsible for this effect have not been completely elucidated. Recently, cAMPindependent, bile activation of CFTR was shown to be present in ileal cells[70]. Like cholangiocytes, ASBT-dependent bile acid uptake was required for CFTR activation but the molecular mechanism for CFTR activation was not disclosed in this study.

In contrast to the stimulatory effects of taurocholate and taurolithocholate, ursodeoxycholate inhibits secretin-stimulated cAMP synthesis and Cl^-/HCO_3^- exchanger activity in isolated cholangiocytes and secretin-stimulated ductal bile flow *in vivo*[8]. Inhibition of cholangiocyte secretion by ursodeoxycholic acid was found to be dependent on the ability of ursodeoxycholic acid to increase intracellular calcium and activate PKCalpha in cholangiocytes[8]. We have proposed that the stimulatory and inhibitory effects of taurocholate and ursodeoxycholate, respectively on cholangiocyte secretion is due to their differing ability to activate intracellular Ca^{2+} and to activate different PKC isoforms[8].

Bile acid signaling of cholangiocyte proliferation

In vitro, taurocholate and taurolithocholate in 1-20 μmol/L concentrations increase H_3-histone expression (a maker of cholangiocyte proliferation)[12]. Feeding taurocholate or taurolithocholate to rats increases ^{3}H-thymindine uptake in cholangiocytes, increases bile duct mass 2 to 3 fold[11] and consistent with a ductal hyperplasia there is accentuation of secretin-stimulated cAMP synthesis and ductal bile flow. The effects of taurocholate and taurolithocholate on bile duct proliferation in the bile acid feeding models occur in the absence hepatic inflammation[11]. Recent studies[68] show that taurocholate can induce phosphorylation of EGF receptor in cholangiocytes, similar to that reported in hepatocytes[64] and the transactivation of the EGF receptor requires transforming growth factor alpha and matrix metalloproteinase[71].

In contrast to taurocholate and taurolithocholate, ursodeoxycholate inhibits cholangiocyte proliferation both *in vitro* in isolated cholangiocytes and *in vivo* in bile duct

ligated rats[8]. The inhibition of cholangiocyte proliferation was found to be dependent on activation of PKCalpha and calcium-dependent pathways[8]. The inhibitory effect of ursodeoxycholate on cholangiocyte proliferation may be one mechanism for the histological and biochemical improvement of diseases targeting the biliary tree (e.g. primary biliary cirrhosis) with ursodeoxycholate treatment. Inhibition of cholangiocyte proliferation may reduce the number of proliferating cholangiocytes that release proinflammatory cytokines[72] or profibrotic signaling molecules such as platelet-derived growth factor[73]. The lack of therapeutic effect for ursodeoxycholic acid in the late stage primary biliary cirrhosis may be at least partially related to lack of proliferating ducts (e.g. ductopenia) as the disease progresses[74]. In a cholangiocarcinoma cell line, we demonstrated that ursodeoxycholic acid inhibits growth by inhibition of Raf through PKC-dependent mechanism[13]. This study supports the need for clinical trials examining the effect of ursodeoxycholic acid in the promotion and progression of cholangiocarcinoma in patients with primary sclerosing cholangitis.

Bile acid signaling of cholangiocyte death and survival pathways

Previous studies in hepatocytes have shown bile acids may be either cytotoxic[75-78] or cytoprotective[79]. Bile acid cytotoxicity may be induced by abrupt permeability of the inner mitochondrial membrane to ions leading to mitochondrial membrane permeability transition (MMPT), depolarization of the mitochondrial membrane potential and uncoupling of oxidative phosphorylation[80]. The uncoupling of oxidative phosphorylation, if extensive, results in ATP depletion and cellular death by necrosis[80]. Furthermore, the associated mitochondrial swelling has also been linked to redistribution of cytochrome c from the intermembrane space to the cytosol. In the cytosol, cytochrome c interacts with apoptotic protease-activating factor 1 to activate caspase 9 and subsequently to cause apoptosis[81]. Bile salt-induced hepatocyte apoptosis also entails activation of the Fas death-receptor and subsequent activation of caspase 8 followed by activation of Bid which leads to mitochondrial dysfunction[75].

Alternatively, bile acids may provide cytoprotective effects. Heuman *et al*[82] proposed that the protective effect of ursodeoxycholic acid in opposing the hepatotoxicity of bile acids was due to its direct interaction with plasma membranes of hepatocytes. Ursodeoxycholic acid may provide membrane stability *via* a physicochemical effect by reducing the toxic bile salt disruption of cholesterol-rich model membranes[82]. More recent studies, revealed that ursodeoxycholic acid does not directly stabilize membranes but rather prevents hydrophobic bile acid-induced membrane disruption by alteration of the structure and composition of mixed micelles[83].

In cholangiocytes, Benedetti *et al*[78] showed that *in vitro*, unconjugated but not conjugated bile acids induce ultrastructural evidence of cytotoxicity. These findings were not observed *in vivo*. Even taurine depleted livers did not show evidence of cytotoxicity despite having high concentration of unconjugated bile acids in bile[78]. The authors concluded that bile acid toxicity, although potentially present in biliary epithelium, is prevented in the intact liver by the presence of active bile acid transport in cholangiocytes.

Our studies have shown that taurocholate feeding is protective against cholangiocyte apoptosis induced by either CCl_4[16] or vagotomy[15]. In CCl_4 treated animals, cholangiocyte apoptosis, demonstrated by the presence of nuclear fragmentation, positive annexin staining and loss of cholangiocyte function (secretin-stimulated cAMP synthesis), was not observed in CCl_4-treated rats that were fed taurocholate[16]. Similarly, CCl_4-induced apoptosis *in vitro* was ablated by pretreating cholangiocytes with 20 μmol/L taurocholate. The taurocholate inhibition of CCl_4-induced apoptosis required activation of PKCalpha[16]. Taurocholate feeding also prevents vagotomy induced cholangiocyte apoptosis[15]. In the vagotomy-induced model, apoptosis is associated with loss of PI3K activity and activation of caspase activities[15]. Taurocholate feeding prevented cholangiocyte apoptosis, loss of PI3K activity and activation of caspase activity. Thus, taurocholate is protective against CCl_4- and vagotomy-induced cholangiocyte apoptosis by activation of the PKC and PI3K-dependent pathways, respectively.

Feeding ursodeoxycholate inhibits the ductal hyperplasia in BDL rats, however these studies showed that the effect of ursodeoxycholate was on inhibition of proliferation without increasing cholangiocyte apoptosis[8]. In contrast, Que *et al*[84] showed that ursodeoxycholate inhibits beauvericin-induced apoptosis in a cholangiocarcinoma cell line and that the inhibition was dependent on preventing cytochrome C release from mitochondria and subsequent activation of caspases.

BILE ACID SYNTHESIS AND CONJUGATION IN CHOLANGIOCYTES

Bile acids, phospholipids and cholesterol are synthesized in a cholangiocarcinoma cell line[85]. Cholangiocytes have also been shown to conjugate bile acids[85]. The contribution of cholangiocyte bile acid synthesis and conjugation to the over all bile acid pool seems minimal since less than 3 percent of the total liver mass is composed of cholangiocytes[86].

Our preliminary data suggests that bile acid synthesis through the alternative (mitochondrial) pathway by cholesterol 27-hydroxylase (Cyp27) maybe important in the regulation of cholesterol transport and prevent cholesterol toxicity due to oxysterols in cholangiocytes[87]. Oxysterols (which increase with a higher cell cholesterol pool) are cytotoxic to smooth muscle and endothelial cells and are potentially carcinogenic.

Oxysterols are present in bile, have been shown to induce cholangiocyte apoptosis and may be carcinogenic in biliary epithelium by increasing expression of COX-2[88]. The mechanisms for preventing oxysterol lipotoxicity in cholangiocytes are unknown. Our preliminary studies show normal rat cholangiocytes express the cholesterol transporters ATP binding cassette subclass A (ABCA1) on the basolateral membrane and Niemann-Pick C1 Like

1 (NPC1L1) on the apical membrane[87,89]. The nuclear receptors involved in sensing lipids liver X receptor β (LXRβ) and peroxisome proliferator-activated receptor δ (PPARδ) and Cyp27 are abundantly expressed in cholangiocytes[87]. Activation of LXRβ, PPARδ, or both in cholangiocytes induces ABCA1 gene and protein expression and increases basolateral excretion of cholesterol. Added oxysterols activate LXR expression and increase basolateral membrane cholesterol efflux. Elevated bile acid levels decrease CYP27 expression. Cholangiocytes absorption of cholesterol at the apical membrane is dependent on the expression of NPC1L1, a cholesterol transporter recently shown to be required for cholesterol absorption in the intestine. Like ABCA1, NPC1L1 is upregulated by PPARδ ligands. We propose that upregulation of CYP27 leads to production of oxysterols, which then activate LXR and cholesterol efflux in cholangiocytes. These studies identify a potential reverse cholesterol transport pathway in cholangiocytes regulated by the cholangiocyte cholesterol and bile acids pools. We propose, like the reverse cholesterol transport involving macrophages, cholesterol or oxysterols taken up from bile by cholangiocytes is released into the circulation return to hepatocytes for elimination. The cholangiocyte reverse cholesterol transport pathway may function to prevent biliary damage due to oxysterols.

GALLBLADDER EPITHELIAL CELLS

Gallbladder epithelial cells and cholangiocytes share a number of functions, however their primary role in the liver and their reaction to disease are quite different. Similar to cholangiocytes, gallbladder epithelial cells express ASBT, and taurocholate have been shown to increase intracellular calcium, activate chloride channels and stimulate mucin secretion[90]. In contrast, ursodeoxycholate, through a PKCalpha-dependent pathway inhibits gallbladder mucin production[90]. The authors proposed that ursodeoxycholate inhibition of gallbladder mucin could provide benefit to biliary disorders such as cystic fibrosis.

BILE ACID EFFECTS ON CHOLANGIOCYTE FUNCTION OR DYSFUNCTION IN HUMANS

Compared to the understanding of the effects of bile acids on rodent cholangiocytes, very little is known regarding bile acid signaling of cholangiocyte function in health and disease in humans. Previous studies of biliary bicarbonate secretion in humans, by employing PET scanning, show that biliary bicarbonate secretion is reduced in primary biliary cirrhosis, the prototypic disease of bile duct damage in humans[91]. After treatment with ursodeoxycholate, biliary bicarbonate secretion in primary biliary cirrhosis patients is increased compared to the pretreatment values[91]. It is not clear why ursodeoxycholic acid inhibits cholangiocyte secretion in bile duct ligated rats but increases cholangiocyte secretion in primary biliary cirrhosis in humans. It is likely that the pathophysiology of these two forms of biliary injury is different.

Cystic fibrosis also targets biliary epithelium in the liver[92]. Clinical studies have shown that ursodeoxycholate may improve liver tests in cystic fibrosis patients[93]. It has been proposed that the mechanism for action of ursodeoxycholate in cystic fibrosis is increased ductal bile flow [as demonstrated by the opening of chloride channels by Shimokura *et al*[66]] that reduces the bile plugs and obstruction due to thick biliary secretions[94]. Ursodeoxycholate has been found to have measurable clinical effects in other diseases that target biliary epithelium (graft versus host disease involving the liver, liver allograft rejection and bile-duct paucity syndromes)[95]. Finally, ursodeoxycholate has been used in other chronic cholestatic liver disorders where biliary epithelium is not the primary target (intrahepatic cholestasis of pregnancy, progressive familial intrahepatic cholestasis, non alcoholic steatohepatitis, alcoholic liver disease, autoimmune hepatitis)[95]. The potential therapeutic effect of ursodeoxycholate in human liver diseases is reviewed elsewhere[95].

In summary, bile acids interact with cholangiocytes in numerous ways. A specific bile acid transporter (ASBT) is localized on the apical membrane, posed to absorb biliary bile acids[5,17]. On the basolateral membrane, four transport systems have been identified (*t*-ASBT, MDR3, an anion exchanger system and the Ostα-Ostβ heteromeric transporter)[6,28,29]. Studies in cultured cholangiocytes show that cholangiocytes transport bile acids from the apical to the basolateral membrane[17]. Indirect evidence for a cholehepatic shunt pathway initiated by bile acid absorption from bile by ASBT that leads to bile acids return *via* the peribiliary plexus to hepatocytes for secretion into bile[7,34,36-38,41]. The contribution of the cholehepatic shunt pathway in overall hepatobiliary transport of bile acids and the role of the cholehepatic shunt pathway in the adaptation to chronic cholestasis due to extrahepatic obstruction remains to be determined. ASBT is both acutely regulated by a cAMP-dependent translocation to the apical membrane[38] and chronically regulated by changes in gene expression in response to biliary bile acid concentration and ubiquitination-dependent proteasome disposal[9]. Biliary bile acid concentration and composition may regulate cholangiocyte functions. After uptake by ASBT, bile acids signal calcium, PKC, PI3K, MEK and ERK intracellular signals in cholangiocytes with resultant changes in cholangiocyte secretion, proliferation and survival[11,12,59,61,66,96]. Different bile acids have differential effects on cholangiocyte intracellular signals, resulting in opposite effects on cholangiocyte secretion, proliferation and survival[12,96,97].

In future studies, the mechanisms explaining how bile acids with different structures can differentially regulate different intracellular signals will be determined. To address the question of how chronic cholestatic liver disease may adapt by changes in cholehepatic shunting, new experimental paradigms to directly quantify bile acid absorption in bile ducts in experimental animals will be developed. Since multiple transporters with varying substrate specificity are present in the sinusoidal and canalicular membrane of hepatocytes, additional bile acid transporters may be found in cholangiocytes. When the mechanisms for bile acid cytoprotective effects in cholangiocytes are defined, a new therapeutic window

in human biliary disorders may open that operates through modulation of biliary bile acid concentration and composition. Finally, the role of cholangiocyte bile acid transport in the promotion or adaptation to human liver disease needs to be determined.

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REVIEW

Synchronous and metachronous occurrence of gastric adenocarcinoma and gastric lymphoma: A review of the literature

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Abstract

The occurrence of both primary gastric lymphoma and gastric adenocarcinoma in the same patient is a rare entity. The possible causative factors of synchronous or metachronous occurrence of both malignancies and varieties in the treatment modalities are reviewed according to published cases in English language medical literature.

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Key words: Gastric adenocarcinoma; Gastric lymphoma; Synchronous occurrence; Metachronous occurrence

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INTRODUCTION

Secondary malignancies associated with Hodgkin's or non-Hodgkin's lymphoma are commonly detected in relation with the improvement in overall survival rate of these patients. The most common type of secondary malignancy seen with lymphoma is acute myelogenous leukemia, however, epithelial malignancies are also reported[1-3]. Intensive radiotherapy and chemotherapy administered to these patients have raised questions about the long-term effects of these therapeutic modalities on the development of secondary malignancies. Primary gastric lymphoma is a relatively uncommon malignancy, occurring in only 1%-7% of all malignant neoplasms of the stomach[4]. The occurrence of both primary gastric lymphoma and gastric adenocarcinoma in the same patient is a rare entity. We review the possible causative factors of synchronous or metachronous occurrence of both malignancies and varieties in the treatment modalities according to published cases in English language medical literature.

INCIDENCE

Since the first description of multiple gastric cancers by *Barth* in 1855[5], case reports of benign and malignant multiple primary tumors have been appearing with an increasing frequency. The incidence of multiple simultaneous gastric cancers is high, ranging from 3.4% to 67.4%[6]. In the first published case series in Germany, authors did not mention the incidence of simultaneous occurrence of malignant lymphoma and adenocarcinoma in the stomach[7]. Thereafter, case series from Japan appeared in the literature. Kasahara *et al*[8] reviewed data of 35 Japanese patients without giving incidence rate for synchronous occurrence. Noda *et al*[9] found an association of both tumors in 2 of 2438 (0.08%) gastric carcinoma cases. The incidence of simultaneous occurrence was 3.7% (9/247) in primary gastric lymphomas and 0.098% (9/9150) in gastric carcinomas in series by Nakamura *et al*[10]. Despite definite expression of incidence rate for synchronous cases, the incidence of metachronous occurrence of both tumors has not been mentioned in the literature.

SYNCHRONOUS CASES

Since the first case reported by Rabinovitch *et al*[11], 56 cases of synchronous occurrence of gastric adenocarcinoma and lymphoma have been published in English language medical literature[7-31]. Majority of these reports included detailed analysis of histopathologic features of tumors instead of clinical follow-up of patients. Basic characteristics of the patients are summarized on Table 1. Twenty-three cases were from the Eastern and 33 cases were from the Western countries. The overall median age was 66 (range 27-85) years. The median age for the Eastern population was 67 and for the Western population was 72 years. Male predominance was observed in both the Western (1.75:1) and the Eastern (3:1) populations. Overall, 62.5% of patients had early gastric carcinoma (EGC) and 30.4% of pa-

Table 1 Synchronous cases reported in the literature

Reference	Age/sex	Lymphoma type	*H pylori*	Adenocarcinoma type	Therapy	Survival months
Rabinovitch[11]	64/m	Lymphosarcoma	NM	EGC	S. gastrectomy	NM
Jernstrom[12]	72/f	Lymphocytic lymphosarcoma	NM	AGC, I, W	S. gastrectomy and 750 R radiotherapy	10
Manier[13]	65/m	Histiocytic	NM	EGC, P	T. gastrectomy and chemotherapy	24
	72/m	Histiocytic	NM	AGC, P	T. gastrectomy	1.5
Lin[14]	56/m	Diffuse lymphocytic	NM	EGC, W	T. gastrectomy and 4500 cGy Co60 radiotherapy	24
Kane[15]	74/m	Diffuse lymphocytic	NM	EGC, P	S. gastrectomy, radiotherapy and chemotherapy	12
Planker[7]	65/m	Immunocytoma	NM	EGC, I, M	T. gastrectomy, splenectomy and 4000 cGy Co60 radiotherapy	30
Czerniak[16]	74/m	Mix type	NM	EGC, W,	S. gastrectomy, radiotherapy and chemotherapy	12
Kasahara[8]	77/m	Small cleaved	NM	AGC, W	S. gastrectomy	24
Noda[9]	61/m	Diffuse large cell	NM	EGC, W	T. gastrectomy and chemotherapy	9
Akosa[17]	79/m	MALT, LG	NM	EGC, M	No treatment	NM
Kelly[18]	85/m	MALT, LG	+	?, I, M	No treatment	2
Von Herbay[19]	79/f	MALT, LG + HG	+	EGC, I, W	T. gastrectomy	
Wotherspoon[20]	55/f	MALT, LG	+	AGC, P	NM	NM
	55/f	MALT, LG + HG	+	EGC, W	NM	NM
	NM/m	MALT, LG	-	AGC, W	NM	NM
	60/f	MALT, LG	+	AGC, W	NM	NM
	34/f	MALT, LG	+	AGC, P	NM	NM
	67/f	MALT, LG	+	EGC, W	NM	NM
	55/f	MALT, LG	+	EGC, W	NM	NM
	55/f	MALT, LG	-	EGC, P	NM	NM
	69/f	MALT, LG	+	AGC, W	NM	NM
Nishino[21]	71/m	Diffuse, large cell type	NM	EGC, tubular type, W	T. gastrectomy, splenectomy and cyclophosphamide, oncovin, prednisolone chemotherapy	120
Hardman[22]	56/m	MALT, LG	+	AGC, Signet-ring cell, P	T. gastrectomy and etoposide, cisplatin, adriamycin chemotherapy	NM
Nakamura[10]	27/m	MALT, LG	+	EGC, I, W	NM	57
	38/m	MALT, LG	+	EGC, D, P	NM	45
	70/m	MALT, HG	+	EGC, I, W	NM	81
	72/m	MALT, LG	+	AGC, I, W	NM	1
	75/f	MALT, LG	+	EGC, I, W	NM	13
	53/m	MALT, LG	+	EGC, I, W	NM	67
	67/f	MALT,LG	+	EGC, I, W	NM	31
	42/m	Immunoblastic	+	EGC, D, P	NM	91
	47/m	MALT, LG	+	EGC, D, P	NM	24
	78/m	T-cell pleomorphic	+	AGC, D, P	NM	1
Ishihama[23]	68/m	Diffuse, small cleaved	+	EGC, W	T. gastrectomy	NM
	61/m	Diffuse, large cell	+	EGC, P	T. gastrectomy	NM
	61/f	Diffuse, lymphocytic	+	EGC, P	S. gastrectomy	132
	77/m	Diffuse, large cell	+	EGC, W	Vincristine,endoxanpredonine, adriamicin chemotherapy and endoscopic mucosectomy	NM
Goteri[24]	51/M	MALT, LG + HG	+	EGC, I, W	NM	122
	55/f	MALT, LG	+	EGC, D, P	NM	33
	80/m	MALT, LG	-	EGC, I, W	NM	12
	57/m	MALT, LG	+	EGC, I, W	NM	10
	53/m	MALT, LG	+	AGC, I, W	NM	3
	66/m	MALT, LG	-	AGC, D, P	NM	10
	69/m	MALT, LG	-	AGC, D, P	NM	8
	69/m	MALT, LG	-	AGC, M, M	NM	3
Kanamoto[25]	47/m	MALT, LG	+	EGC, D, P	T. gastrectomy	24
Montalban[26]	77/m	MALT, LG	NM	GC	NM	NM
	68/f	MALT, HG	NM	GC	NM	NM
Cammarota[27]	47/m	MALT, LG	+	AGC, I, P	Etoposide, epirubicin, cisplatin chemotherapy and T. gastrectomy	NM
Chan[28]	71/m	MALT, LG	-	EGC, P	S. gastrectomy	NM
	58/f	MALT, HG	+	EGC, P	S. gastrectomy	NM
	75/f	MALT, HG	+	EGC, P	S. gastrectomy	NM
Kafes[29]	78/m	MALT, NM	+	EGC, I, P + stromal tumor	T. gastrectomy	20
Sakai[30]	51/f	MALT, LG	+	EGC, tubular, M	T. gastrectomy	NM
Suenaga[31]	73/m	MALT, LG	-	AGC, I, W	S. gastrectomy, D3 dissection	23

m: male, f: female, NM: not mentioned, EGC: early gastric carcinoma, AGC: advanced gastric carcinoma, GC: gastric carcinoma, I: intestinal type, D: diffuse type, W: well differentiated, P: poor differentiated, M: moderately differentiated, MALT: mucosa-associated lymphoid tissue, HG: high grade, LG: low grade, S. gastrectomy: subtotal gastrectomy, T. gastrectomy: total gastrectomy.

tients had advanced gastric carcinoma (AGC), the type of gastric carcinoma was undefined in the rest of the patients. EGC constituted a majority of the Eastern cases (82%), whereas 48% of cases were from the Western countries. Before the report by Kelly *et al*[18] in 1994, the presence of *H pylori* was not mentioned by the authors. Since then, reported *H pylori* infection rate reached 79%. The infection rate in the East (86%) was higher than that in the West (72%). The prevalence of *H pylori* was higher in EGC (86%) than that in AGC (64%). There was no significant correlation between the prevalence of *H pylori* and the differentiation of adenocarcinoma. The declared results of adenocarcinoma histopathology were not uniform in relation to the variations in the classification systems used by the authors. However, differentiation of the tumor was clearly revealed by all authors. Poor and well differentiated adenocarcinomas showed an equal distribution in the Eastern (43.5% and 52%, respectively) and the Western cases (42% and 42%, respectively).

Majority of the lymphomas were MALT (mucosa-associated lymphoid tissue) type lymphoma (69.6%) and low grade one (87.2%). The association of *H pylori* with MALToma was 86% in the Eastern cases and 72% in the Western cases. Six of 7 (86%) patients with other types of gastric lymphoma associated with gastric adenocarcinoma were *H pylori*-positive. There was no data about the size of the tumor found in 32% of the cases. The size of the lymphoma was larger than carcinoma in 57% of cases. Overall, lymph node metastasis was found in 41% of the cases. In patients with early carcinoma and positive lymph node metastasis, the origin of metastatic lymph node was usually lymphoma (89%). In the presence of advanced carcinoma, metastases originated from the adenocarcinoma, either alone or in combination with the lymphoma. The topographic interrelation of both tumors was also revealed in the reports (n = 53/56). Majority of the cases (n = 29, 54.7%) had independent tumors. There was no significant difference between the Eastern and the Western cases in this respect. Collision of both tumors was reported in 14 (26.4%) cases. There were 4 cases with contiguous and 5 cases with intermingling tumors.

METACHRONOUS CASES

In contrast to synchronous occurrence of gastric adenocarcinoma and lymphoma, majority of cases (90%, 27/30) with metachronous occurrence of both tumors were reported from the Western countries[10,32-47]. In 1950, McNeer *et al*[32] published the first case in the literature. The features of the 30 patients are summarized in Table 2. The median age was 50 (8-82) years. Of these patients, 18 were male and 12 were female. Previously detected malignancy in 28 patients was lymphoma. Only 2 cases reported by Nakamura *et al*[10] were diagnosed as lymphoma after the treatment of gastric adenocarcinoma. One of these tumors developed in the remnant stomach in the 7th year after subtotal gastrectomy and the other developed in the 13th month after endoscopic mucosal resection. Rest of the patients suffered from various types of lymphoma previously. Leading type of lymphoma was MALT lymphoma (30%), followed by large cell lymphoma (20%). The existence of *H pylori* was not mentioned in the reports published before 1997. Thereafter, 9 of 10 reported cases were found infected with *H pylori*. Median interval between the treatment of lymphoma and the diagnosis of gastric adenocarcinoma was 90 (6-408) mo. The reported interval after the treatment of MALToma (median 27, range 6-108 mo) was shorter than the other types of lymphoma (median 120, range 42-408 mo). Adequate description of histopathology of adenocarcinoma was determined in 26 of 30 cases. After the latent period, majority of cases suffered from advanced gastric adenocarcinomas (65%) and 9 patients had EGC.

PATHOLOGIC ASPECTS OF SYNCHRONOUS OCCURRENCE

The relation of both tumors in the stomach was classified in four categories: (1) separate tumors; (2) collision tumor; (3) contagious tumor, without any intermingling between malignant components; and (4) intermingling (admixture) of both tumors. Histological classification of gastric lymphomas is based on systems originally designed for nodal lymphomas as Musshoff modification of the Ann Arbor staging system, Working formulation or Kiel classification. However, these are not optimal for the documentation of specific relevant features of primary extranodal lymphoma in the gastrointestinal tract. Several modifications on TNM staging system and alternatives have been proposed[48,49]. Recently, the European Gastro-Intestinal Lymphoma Study Group (EGILS) proposed a new modification of TNM staging system for the gastric lymphomas[50]. In 1983, the histologic relationship of extranodal B-cell lymphomas and MALToma was recognized by Isaacson and Wright, and classification as a separate entity of MALT-NHL was proposed - the MALT lymphoma concept[51,52]. However, the first report of synchronous occurrence of MALToma with gastric adenocarcinoma appeared in the literature in 1990. Metachronous occurrence of MALToma was reported at the end of 1990's. Therefore, it is easily speculated that authors are affected by the classification systems for nodal lymphomas before 1990.

Histopathological disorders of gastric mucosa are not specific for any neoplasm, but intestinal-type adenocarcinomas frequently showed atrophy, intestinal metaplasia, and not uncommonly, dysplasia of the surrounding non-neoplastic gastric mucosa. Diffuse-type adenocarcinomas did not frequently show such lesions. Primary lymphomas displayed expansive lymphoid follicles and also a high percentage of atrophy and intestinal metaplasia of the surrounding gastric mucosa[53]. In addition to the similar histopathological findings in the surrounding gastric mucosa of intestinal-type adenocarcinoma and primary gastric lymphoma, lymphocytic gastritis was found more frequently in patients with gastric carcinoma and primary gastric lymphoma than in unselected patients undergoing endoscopy[54]. This finding suggests that these two disparate gastric tumors may share an immunological dysfunction or a common pathogenesis. Zamboni *et al*[55] pointed out that foveolar lymphoepithelial lesions or signet-ring cells were found in 37% of MALT lymphomas. Like in MALT

Table 2 Metachronous cases reported in the literature

Reference	Age/sex	Lymphoma type	Lymphoma therapy	Interval	Adenocarcinoma therapy
McNeer[32]	27/f	Reticulum cell sarcoma	S. gastrectomy; radiotherapy for local recurrence	6.5 yr	T. gastrectomy
Fleischer and Walker[33]	61/m	Lymphosarcoma	S. gastrectomy; 4000 cGy	5 yr	Supportive treatment
Bockus[34]	49/m	Lymphosarcoma	Radiotherapy	3.5 yr	T. gastrectomy
Komarov[35]	40/m	Round cell sarcoma	S. gastrectomy	19 yr	NM
Morgenstern[36]	46/f	Reticulum cell sarcoma	S. gastrectomy	25 yr	Resection
Ettinger and Carter[37]	55/m	Lymphosarcoma	S. gastrectomy; 2000-2600 cGy	16 yr	Palliative surgery
Shani[38]	47/m	Reticulum cell sarcoma	S. gastrectomy	31 yr	Resection
	29/m	Diffuse, poorly differentiated lymphocytic	S. gastrectomy; orthovoltage for whole abdomen (6 d)	34 yr	Exploration
	51/f	Diffuse, mixed lymphocytic histiocytic	S. gastrectomy; 1800 cGy X-R + 2000 cGy Co 60. [1]2030 cGy for recurrence (16 yr later)	16 yr	Exploration
	48/m	Diffuse, poorly differentiated lymphocytic	S. gastrectomy; 3000 cGy.	10 yr	Exploration; combined chemotherapy
Sellin[39]	45/m	Diffuse, large cleaved cell	2300 cGy; S. gastrectomy; 4000 cGy (4 yr later)	13 yr	Total gastrectomy
Brumback[40]	8/m	Diffuse undifferentiated (small noncleaved)	S. gastrectomy; cyclophosphamide, vincristine, prednisolone for 1 mo; 4075 cGy; 6-mercaptopurine, vincristine, prednisolone, methotrexate for 3 yr	6 yr	Gastrojejunal bypass; 5-FU, doxorubicine, mitomycin chemotherapy
	15/m	Hodgkin's disease, nodular sclerosis type	3500 cGy to upper abdomen; 2000 cGy to left axillary-cervical-supraclavicular; cyclophosphamide, vincristine, procarbazine, prednisolone, adriamycin for 2 yr	10 yr	NM
Baron[41]	58/m	Diffuse large cell	3150 cGy to upper abdomen	10 yr	S. gastrectomy
	24/f	Reticulum cell sarcoma	S. gastrectomy; 4000 cGy	15 yr	No treatment
	60/m	Diffuse large cell	S. gastrectomy; cyclophosphamide, vincristine, prednisolone, doxorubicin chemotherapy	4 yr	T. gastrectomy and S. esophagectomy
	56/m	Well differentiated lymphocytic	S. gastrectomy; 3700 cGy; oral cyclophosphamide chemotherapy	12 yr	T. gastrectomy
Zorlu[42]	43/m	Diffuse, large cleaved cell	S. gastrectomy; cyclophosphamide, vincristine, prednisolone (2 courses); 4500 cGy; cyclophosphamide, vincristine, prednisolone (4 courses)	8 yr	Near total gastrectomy; 5-FU, mitomycin
	35/f	Diffuse, large cleaved cell	S. gastrectomy; 4000 cGy; cyclophosphamide, vincristine, prednisolone (6 courses)	8 yr	T. gastrectomy
Nakamura[10][1]	69/f	Immunoblastic	NM	7 yr	S.gastrectomy
	82/f	MALT, HG	NM	13 mo	Endoscopic mucosal resection
Zauber[43]	78/f	Large cleaved cell	S. gastrectomy; 4500 cGy; i.v. cyclophosphamide chemotherapy (9 courses)	4 yr	T. gastrectomy
Montalban[26]	42/m	MALT	Cyclophosphamide, doxorubicin, vincristine, prednisone	108 mo	NM
Hasegawa[44]	72/f	MALT	Antibiotic therapy for *H pylori*	6 mo	S. gastrectomy
Morgner[45]	74/m	MALT, LG	Antibiotic therapy for *H pylori*	4 yr	Endoscopic mucosal resection
	70/m	MALT, LG	Antibiotic therapy for *H pylori*	5 yr	Endoscopic mucosal resection and argon plasma coagulation
	77/f	MALT, LG	Antibiotic therapy for *H pylori*	4 yr	Endoscopic mucosal resection
Ghosdal[46]	32/m	MALT, LG	S. gastrectomy; Antibiotic therapy for H pylori (for 2 wk);	15 mo	No treatment
Raderer[47]	67/f	MALT	Antibiotic therapy for *H pylori* (for 9 mo)	9 mo	S. gastrectomy
	61/f	MALT	Antibiotic therapy for *H pylori* (for 14 mo); 2CdA chemotherapy (4 courses)	27 mo	S. gastrectomy

m: male, f: female, NM: not mentioned, MALT: mucosa-associated lymphoid tissue, HG: high grade, LG: low grade, S. gastrectomy: subtotal gastrectomy, T. gastrectomy: total gastrectomy, [1]Only 2 cases reported by Nakamura *et al* were diagnosed as lymphoma after the treatment of gastric adenocarcinoma.

lymphomas, gastric carcinomas are invariably accompanied by lymphoid proliferations ranging from reactive lymphoid follicles to MALT lymphomas[56]. These observations also supplied the theory of common pathogenesis of MALT-type lymphoma and gastric carcinoma.

The incidence of early gastric carcinoma in synchronous tumors was remarkably high. Especially in the Eastern countries, routine screening for gastric carcinoma is practiced, and up to about 50% of gastric carcinoma diagnosed in the early stage[57]. This observation, together

with the finding that most lymphomas were larger than adenocarcinomas, suggested that lymphomas might develop before adenocarcinomas or that the presence of MALT lymphoma might increase the risk of developing gastric carcinoma.

ETIOLOGIC FACTORS OF SYNCHRONOUS OCCURRENCE

H pylori

It is tempting to hypothesize that *H pylori* is a common etiological agent for synchronous occurrence of gastric adenocarcinoma and primary gastric lymphoma. Since 1994, the existence of *H pylori* has been investigated in almost every report. In the present analysis, in areas with high prevalence (Eastern) and low prevalence (Western) of *H pylori*, synchronous tumors are associated with *H pylori* infection rates of 86% and 72%, respectively, and are similar to or higher than the reported infection rates of isolated gastric adenocarcinoma and isolated MALT lymphoma or other primary gastric non-hodgkin lymphoma[20,58-60]. Therefore, *H pylori* may have an important etiological role for synchronous tumors in both high and low prevalence areas.

H pylori plays a key role in the natural history of gastric MALT lymphoma, representing an example of antigen-mediated tissue stimulation and lymphoproliferation, with possible subsequent lymphomagenesis[58,61]. Antigenic mimicry between *H pylori* and the host mucosa was held responsible for inducing autoimmune responses which lead to development of the disease[62,63]. Kawahara *et al*[64] found that the increase of antibody titers to HCG-27 cells in *H pylori*-positive patients with MALT lymphoma compared to titers in patients with other gastroduodenal diseases and in healthy subjects. Lymphoid follicles which are not present in the normal stomach show development in the setting of chronic gastritis. Genta *et al*[65] have observed a strong correlation between follicular gastritis and *H pylori* infection. Wotherspoo*n et al*[58] suggested that *H pylori* might trigger the acquisition of MALT in the gastric mucosa, and this lymphoid tissue is thought to harbor the precursor cells in MALT-NHL. These precursor cells change gradually into malignant lymphoma cells with autonomous and uncontrolled growth by accumulation of genetic alterations such as mutations, deletions, and amplifications. *In vitro* experiments have demonstrated that in the earlier phases of development, the growth of genetically altered lymphoid cell is not yet fully autonomous, and proliferation partly depends on *H pylori*-related proteins and T cells[66-69]. On the other hand, *H pylori* infection has been linked to the intestinal-type gastric adenocarcinoma through a chain of events that starts as acute gastritis and progresses to chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and eventually, adenocarcinoma formation[70,71]. A recent meta-analysis has revealed that *H pylori* infection is associated with a 2-fold increase in developing gastric adenocarcinoma[72]. These epidemiologic and pathologic data about the relation of *H pylori* infection with gastric adenocarcinoma and primary gastric lymphoma are supported by a recent genetic study[73]. *H pylori* genotyping on archived gastric tissue revealed that *H pylori* strains with certain combinations of virulence subtypes are associated with gastric carcinoma or MALT lymphoma. Thus, the virulence subtype composition vacA s1a and iceA1 occurs mainly in gastric carcinoma, whereas the vacA s1t/m2 iceA1 combination is found in MALT lymphoma[73]. Therefore, in the future studies, the composition of virulence subtypes might be taken into account during the evaluation of possible sequelae of gastric mucosal damage caused by *H pylori* infection.

Epstein-Barr virus

Studies of Epstein-Barr virus (EBV) suggest that an aberrant antibody response to infection may occur years before the appearance of a tumor[74]. The frequency of EBV detection has been reported to be 6%-18% in gastric lymphomas[75-77] and 7%-16% in ordinary gastric adenocarcinomas without lymphoid stroma[78-80]. Whether EBV plays a pathogenic role in either of these tumors is still unclear[81]. Although any mechanism related to EBV in tumorigenesis of gastric malignancies remains highly speculative, it has been demonstrated that there is a delay in apoptosis in EBV-positive gastric carcinomas (associated with up-regulation of BCL-2 and p53) and a decrease in cellular differentiation (associated with decreased E-cadherin expression)[82]. Nakamura *et al*[10] found no specific association between EBV infection and these double malignancies.

Exposure to atomic blast

It is controversial whether the incidence of gastric carcinoma is higher in persons exposed to an atomic blast in comparison to non-exposed subjects[83-85], but Suehiro *et al*[83] suggested that the incidence is the highest in persons exposed within a 2.0-Km radius ground zero. The relation of primary gastric lymphoma and atomic bomb exposure is under debate. Recently, t (11; 18) (q21; q21) and t (1;14) (p22; q32) translocations have been reported to be associated with MALT-type lymphoma. The former translocation results in the formation of fusion protein API2-MALT1, and the latter in the fusion protein pf BCL10 and IgH. These fusion transcripts are thought to be molecular markers for MALT-type lymphoma not responding to *H pylori* eradication and highly correlated with aberrant nuclear BCL10 expression, which may serve as a screening tool for fusion[86-88]. These investigations support the etiologic role of atomic bomb blast on the formation of MALT-type lymphoma.

ETIOLOGIC FACTORS OF METACHRONOUS OCCURRENCE

Epithelial malignancies, the common cancers of adulthood, are detected rarely in the post-treatment course of Hodgkin's disease and other lymphomas[2,3,26]. However, Greene and Wilson[88,89] observed a significant increase in the incidence of gastric carcinoma in the period following the treatment of non-Hodgkin lymphomas.

Previous gastric surgery

Previous gastric surgery is a well known precancerous con-

dition[90,91]. Gastroesophageal reflux and gastritis, formation of nitrosamines due to gastric pH decrease and untreated *H pylori* infection may play a role in carcinogenesis of the gastric remnant[92]. So-called "stump carcinoma" develops between 13-27 years after resection in patients treated with gastrectomy for benign conditions[91].

Radiotherapy and chemotherapy

Though the stomach is considered relatively radio-resistant compared to other parts of the gastrointestinal tract, side effects and complications progressively increased when the total dosage delivered was above 4500 cGy[93,94]. The effects of radiotherapy on the risk of carcinogenesis are well known[95]. Hirose *et al*[96] demonstrated that localized radiation induced gastric adenocarcinoma development in an experimental model. Brown *et al*[97] reported a slight increase in the incidence of gastric adenocarcinoma in patients with ankylosing spondylitis under radiation therapy. In contrast, when the relatively low dose (less than 3400 cGy) radiotherapy was used to decrease acid production in 2049 peptic ulcer patients, only two cases of leiomyosarcoma were reported. The elapsed time between radiotherapy and the diagnosis of these cases were reported as 14 and 26 years[98].

Both series reported by Stanford and Milan concluded that combined administration of chemotherapy and radiotherapy increases the occurrence rate of secondary malignancies[1,2]. In these series, the most common secondary malignancy was especially acute myelogenous leukemia. Its occurrence was associated with the administration of alkylating agents (nitrogen mustard, vincristine, prednisone, and procarbazine in Milan series)[2]. Solid tumors were commonly associated with radiation therapy[1,2]. Brumback *et al*[40] concluded that secondary gastric adenocarcinoma occurred in one of these cases probably due to oral administration of procarbazine for Hodgkin's disease. In addition to these factors, *H pylori* infection was also held accountable for metachronous occurrence of both tumors, as discussed above.

DIFFICULTIES IN DIAGNOSIS

It may be difficult to diagnose the two different tumors in the stomach before surgery. Because most of the patients with gastric adenocarcinoma and coexisting primary gastric lymphoma are associated with *H pylori* infection, the presence of MALT-type or other types of primary gastric lymphoma should be taken into consideration when gastric adenocarcinoma with gastritis is detected on endoscopy. In surgical specimens, not only the lesions of adenocarcinoma but also the background mucosa of cancerous lesions must be examined with regard to infiltration of lymphocytes, and the presence of gastritis, as well as the presence of *H pylori*. Signet-ring cells around MALT lymphomas or lymphoid proliferations around gastric adenocarcinomas should be kept in mind against over-diagnosis during the histopathological examination. Considering the metachronous occurrence of gastric adenocarcinoma and gastric lymphoma, proper endoscopic follow-up should be performed in the remnant stomach, especially when *H pylori* infection is present.

TREATMENT

Treatment modalities for synchronous cases

Treatment of synchronous cases is generally applied according to the presence of adenocarcinoma. Distal or total gastrectomy is performed depending on the tumor localization. Preoperative chemotherapy was preferred only in two cases. Cammarota *et al*[27] decided to administer etoposide, epirubicin, cisplatin chemotherapy according to the presence of locally advanced tumor stage (T3 N1) in laparoscopic exploration. Older patients in the series by Ishihama *et al*[23] were exposed to 10 courses of vincristine, endoxan, predonisdone, adriamicin chemotherapy and endoscopic resection of the tumor. Postoperative chemotherapy was administered only to 6 (10.7%) patients.

Treatment modalities for metachronous cases

Eight of thirty patients underwent subtotal gastrectomy and radiotherapy against lymphoma. Chemotherapy was administered in different combinations with other treatment modalities in 7 patients. Two patients were treated by only chemotherapy. Radiotherapy was performed solely in 2 patients. Overall, 10 cases with lymphoma were treated without surgical treatment. All patients with MALToma were treated with antibiotic regimen against *H pylori*, except cases reported by Nakamura *et al*[10] *and* Montalban *et al*[26], the authors of both did not mention anything about the treatment of *H pylori* infection in their reports. Every patient with *H pylori* infection was eradicated and no one was re-infected or re-colonized during the follow-up period. Except cases with widespread metastasis, total gastrectomy or palliative resections were performed in patients with AGC. Patients with EGC were exposed to more conservative surgery, such as endoscopic mucosal resection[10,45].

Surgery

Clear guidelines for management have not been designed for synchronous adenocarcinoma and primary gastric lymphoma. Traditionally, aggressive surgical resection has been the mainstay of gastric lymphoma treatment because by this treatment modality it would be possible to collect definitive tissues for pathologic examination, allow exploration of the abdomen, reduce the tumor burden and obviate the concern that gastric hemorrhage or perforation would complicate medical treatment of lymphomas. Recently, radical gastrectomy is disputed and considered unnecessary for gastric lymphomas. Lesser procedures are now accepted where resection of the gross disease and involved lymph nodes will provide adequate results[99-102]. Some authors advocate wide resection and extensive lymph node dissection alone for adequate treatment of stage 1E or pure MALT lymphomas with a survival rate of > 95%[103,104]. In a retrospective study from Italy patients in different stages of gastric lymphoma who underwent surgical resection when feasible, the ten-year actuarial survival rates were markedly higher (100% and 80%, respectively) for stage IE and IIE as compared with stage IIIE and IVE (21% and 0%, respectively)[105]. Surgical resection with clear margins for lymphoma is advised in order to maximize the chance of cure[100,106]. However, others have found no difference in survival, whether the margin of resection was

clear or not, as long as post-operative chemotherapy was given[107]. Operative mortality rates for gastric lymphoma reached 25% in cases with advanced tumor stage[108]. Therefore, aggressive surgery for gastric lymphoma is not indicated due to increased morbidity which outweighs the benefit gained in terms of survival[107]. Surgery for gastric lymphoma is now often reserved for patients with localized disease, residual disease after non-surgical therapy or for rare patients with complications[109]. If the coexisting adenocarcinoma is diagnosed correctly, surgical treatment, a first-line therapy, is preferred according to adenocarcinoma treatment principles. Total or subtotal gastrectomy with D1 or D2 dissection must be performed.

In metachronous cases, generally, the occurrence of primary gastric lymphoma precedes gastric adenocarcinoma, resection of the remnant stomach must be done with or without a combination of other treatment modalities. However, a close follow-up of the patients undergoing gastric lymphoma treatment allows early detection of carcinogenesis. Limited endoscopic resections might be an alternative treatment for these cases. Similar to gastric lymphoma as a previous malignancy, patients in whom limited resection is performed for gastric adenocarcinoma should undergo a close endoscopic follow-up. The *H pylori* status with histopathologic alterations in the resected stomach and in the remnant stomach must be detected and followed up for early detection of metachronous occurrence of gastric lymphoma.

Chemotherapy

The effect of chemotherapy as a sole treatment for gastric lymphomas is still under debate. The needs behind trying chemotherapy were the considerable morbidity and mortality associated with resection[108]. Some authors reported better survival rates in patients with primary gastric lymphoma treated by chemotherapy alone or combination with radiotherapy when compared to surgical treatment alone[110,111]. Other reports showed no apparent difference in survival between patients treated by chemotherapy or surgery and chemotherapy with survival rates of 67% and 60%, respectively. The fear of chemotherapy-related complications, for instance, bleeding and perforation, has been disputed, and less significant compared with surgical resection[111,112]. Therefore, chemotherapy has been suggested and adopted as a primary mode of treatment for primary gastric lymphomas. Whereas cyclophoshamide, vincristine and prednisolone were adopted for low grade lymphomas, high grade tumors were treated with doxurubicin, teniposide, cyclophosphamide and prednisolone. The combination of both regimens with surgical resection has increased the survival rates up to 80% and 100%, respectively[113]. In contrast to primary gastric lymphomas, chemotherapy regimens have not been used as a sole therapy in the treatment of gastric adenocarcinoma, except in cases with metastatic disease. Systemic chemotherapy for metastatic disease has a marginal survival benefit when compared with best supportive care, while no standard worldwide regimen has been established yet. In Japan, especially the number of patients treated with endoscopic mucosal resection for early gastric adenocarcinoma is increasing year by year. Chemotherapy may be a more important part of treatment protocols in these patients in near future, because of the increasing survival rates. Cisplatin, 5-fluorouracil and mitomycin C were commonly used in various combinations for patients with gastric adenocarcinoma[114].

Radiotherapy

In most instances, radiotherapy is used as an adjuvant to surgery, chemotherapy or both for primary gastric lymphoma. It has rarely been tried as a single mode of therapy[115,116]. However, limited trials have suggested that radiotherapy can be utilized as a primary mode of treatment with a reasonable outcome[116,117]. Radiotherapy has been studied in comparison with other treatment modalities for stage IE and IIE of primary gastric lymphomas with a comparable outcome of 80%-89% survival[115,118,119]. Radiation was used post-operatively in high- and low-grade lymphomas, for residual tumors in stages I and II to improve the disease-free survival[120]. Combination of radiotherapy with chemotherapy might improve the chance of stomach conservation of these patients which may approach 100%[121]. Contradictory studies have found combined radiotherapy with either resection or chemotherapy no significant difference in both modalities, with a survival rate of 82%-88%[116,119]. Radiation therapy has a limited but well established role in the care of those afflicted with gastric adenocarcinoma. Radiation therapy can provide considerable relief of local gastric cancer symptoms. Approximately 50% to 75% of patients have had symptomatic improvement of problems, such as obstruction, bleeding and pain in a variety of trials[122,123].

Antibiotic therapy

Because gastric MALT lymphomas have a high association with *H pylori* infection, eradication of *H pylori* with antibiotics is very important[58,60,124,125]. Isaacson *et al*[125] suggested that antibiotic eradication of *H pylori* removes the growth stimulus from gastric MALT lymphoma without necessarily eradicating the neoplastic B-cell clone. This clone may re-expand, but in the absence of concomitant re-infection with *H pylori*, this is likely to be a self-limiting event. Short and long-term follow-up results of medical eradication of *H pylori* in patients with gastric MALT lymphoma have been published in the literature[124,125]. However, patients still require periodic surveillance endoscopies and may require more traditional treatment of their lymphoma. Antibiotic therapy may fail to cure gastric lymphomas when there is a bulky tumor with a high-grade component or when the gastric lymphoma is associated with carcinoma[126]. A combination of partial gastrectomy and antibiotic therapy might be an alternative treatment for primary gastric lymphomas. However, periodic surveillance endoscopies are required after partial gastrectomy and antibiotic therapy. There has been no defined role of antibiotic therapy against *H pylori* in the treatment of gastric adenocarcinoma. Eradication of *H pylori* infection can be placed in preventive measures of gastric adenocarcinoma. If *H pylori* infection is detected in the partially resected

stomach secondary to adenocarcinoma, the postoperative antibiotic treatment against *H pylori* may be administered for hindering metachronous occurrence of primary gastric lymphoma.

PROGNOSIS

Reported period of follow-up for synchronous cases ranged from 1 to 132 mo. Overall, data about surveillance of patients were insufficient to perform analysis. Some authors did not mention follow-up data whereas others did not report treatment modalities. However, shorter postoperative survey was observed in cases with AGC (up to 23 mo) when compared to cases with EGC. The follow-up study conducted by Nakamura *et al*[10] revealed that the survival rate of patients with synchronous occurrence of gastric lymphoma and adenocarcinoma appeared to be similar to that for previously reported patients with gastric adenocarcinoma without lymphoma, and was significantly worse than patients with primary gastric lymphoma without adenocarcinoma. As discussed above, majority of metachronous cases suffered from advanced gastric adenocarcinomas after the latent period. Eleven of 17 patients with AGC died within 18 mo. Despite incomplete survival data of cases with EGC, Zauber *et al*[42] reported 96 mo disease-free survival after total gastrectomy. Nakamura *et al*[10] did not reveal any survival data regarding primary gastric lymphoma occurring after treatment of gastric adenocarcinoma.

In summary, with the help of case reports or series, the synchronous or metachronous occurrence of primary gastric lymphoma and gastric adenocarcinoma have evolved in various aspects. Clinicians should be alert for the possible synchronous or metachronous occurrence of both tumors during diagnosis or follow-up of each other. Authors suggest the application of principles for gastric adenocarcinoma treatment to cases with synchronous occurrence. The biologic behavior of the latest diagnosed tumor determines the accurate management of metachronous occurrence of primary gastric lymphoma and gastric adenocarcinoma.

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Hepatocellular carcinoma in Lebanon: Etiology and prognostic factors associated with short-term survival

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Abstract

AIM: To study the epidemiology of HCC in Lebanon and prognostic factors predictive of early mortality.

METHODS: An observational follow-up cohort study of HCC cases diagnosed over a five-year period was carried out. Multivariate analysis was conducted to identify prognostic factors in comparison to Cancer of the Liver Italian Program (CLIP) score. Multiple variables including the etiology of underlying liver disease, the demographic characteristics of patients, and the severity of liver disease evaluated by the Child-Pugh score were studied. Tumor parameters included the time of diagnosis of HCC, alpha-fetoprotein level, number and size of nodules, presence of portal vein thrombosis, and treatment modalities. Death or loss of follow-up was considered as an end-point event.

RESULTS: Ninety-two patients (mean 60.5 ± 22.3 years) were included. Etiology of underlying disease was hepatitis B, C, and alcohol in 67%, 20%, and 23.5% respectively. Child-Pugh class at diagnosis was A, B, and C in 34.8%, 39.3% and 25.8% respectively. Overall survival was 44.8%, 32.8% and 17.6% at 1, 2 and 3 years respectively (mean F/U 40.2 ± 23.5 mo). Multivariate analysis identified three predictors of early mortality (< 6 mo): bilirubin > 3.2 mg/dL (P < 0.01), HCC as first presentation of liver disease (P = 0.035), and creatinine > 1 mg/dL (P = 0.017). A score based on these variables outperformed the CLIP score by Cox proportional hazard. ROC curve showed both models to be equivalent and moderately accurate.

CONCLUSION: HBV is the leading cause of HCC in Lebanon. Independent predictors of early mortality are elevated bilirubin, creatinine and HCC as first manifestation of disease. Prospective validation of a score based on these clinical parameters in predicting short-term survival is needed.

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Key words: Liver; Epidemiology; Hepatitis; Neoplasm; Cancer; Cirrhosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignancy occurring most often in the setting of liver cirrhosis. The causes of the underlying liver disease differ according to the geographical distribution. Common risk factors for HCC include infection with hepatitis B virus (HBV), hepatitis C virus (HCV), cirrhosis of any cause, alcoholic liver disease, and inherited metabolic diseases such as hemochromatosis and α1-antitrypsin deficiency. HCC occurs most commonly in sub-Saharan Africa and parts of the Far East such as China, Taiwan, Korea, Japan, and Vietnam. HCV infection with advanced fibrosis or cirrhosis is the main cause of HCC in Japan, South Africa, Egypt, the United States and Southern Europe[1-4]. In Northern and Central European countries, ethanol is still the leading cause of cirrhosis and is responsible for the majority of HCC cases[5]. There has been no report on the epidemiology of HCC in Lebanon. Epidemiologic data from neighboring countries differ widely with the primary risk factor for HCC attributable to HBV in Turkey[6], HBV/HDV in Jordan[7], and HCV in Saudi Arabia[8,9].

The expected survival of patients with HCC is an important parameter in predicting mortality on a liver transplantation waiting list. The prognostic factors for survival in patients with HCC have been identified in previous studies and sub-classified into 3 groups: (1)

demographic characteristics including age and gender; (2) factors related to HCC such as tumor size, the number of nodules, vascular invasion, the presence of a tumor capsule or metastasis; and (3) factors related to underlying liver disease severity, including ascites, encephalopathy, or serum bilirubin. The Child-Pugh classification, used in patients with cirrhosis, only considers liver disease and not tumor characteristics. Orthotopic liver transplantation (OLT) is an excellent treatment for early hepatocellular carcinoma. There is, however, a paucity of data on survival according to intention-to-treat analysis and on the rate of dropout from the waiting list for OLT among patients with HCC due to shortage of grafts. This is compounded by the fact that up to 30% of eligible patients develop contraindications to the procedure while waiting for a donor. All of the above result in less than optimal prediction of early mortality in HCC patients, particularly in populations who present with advanced disease and where therapeutic options may be limited. The aim of the present study was to describe the epidemiology of HCC in Lebanon, and to identify factors predictive of early mortality in our patients who could seldom benefit from liver transplantation.

MATERIALS AND METHODS

Patients

The study is a multicenter follow-up cohort study consisting of 92 patients diagnosed with HCC between 1998 and 2003. The study involved three university hospitals in Beirut: Hotel-Dieu de France (Université Saint Joseph), American University of Beirut Medical Center, and Saint-Georges Hospital (Balamand University Medical School). Patients were identified from the medical records and from specialty liver clinics. Information regarding deceased patients was obtained from medical records and prospective follow-up was continued for new and existing patients. Multiple variables including the etiology of underlying liver disease, the demographic characteristics of patients including age and gender, and the severity of liver disease evaluated by the Child-Pugh score were studied. Tumor parameters included the time of diagnosis of HCC, alpha-fetoprotein level, number and size of nodules, presence of portal vein thrombosis, and treatment modalities. Death or loss of follow-up was considered as an end-point event.

HCC was diagnosed according to any one of the following criteria: (1) an abnormal liver morphology with a tumor nodule that is enhanced during the arterial phase following intravenous contrast injection on CT-scan or MRI. (2) an elevated alpha fetoprotein level > 400 ng/mL in the presence of a liver nodule, and (3) biopsy-proven HCC[10]. Patients who did not meet one or more of these diagnostic criteria were not included in the study. Cirrhosis was confirmed according to histology and/or by clinical and biochemical signs of hepatic insufficiency, ascites, esophageal varices, and ultrasonographic signs of portal hypertension. Tumor eligibility-for-treatment criteria included: a single nodule of less than 5 cm without evidence of vascular invasion, or less than 3 nodules of < 3 cm each with the absence of portal vein thrombosis.

Table 1 Characteristics of the 92 patients with hepatocellular carcinoma *n* (%)

Age(yr)	At diagnosis of cirrhosis before development of HCC	56.0 ± 26.3
	At HCC diagnosis	60.5 ± 22.3
Gender	Male	78 (84.8)
Etiology	HBV	62 (67.4)
	HCV	18 (19.6)
	HBV + HCV	77 (83.7)
	Alcohol abuse	22 (23.9)
Liver disease severity at time of diagnosis	Child-Pugh A	32 (34.8)
	Platelet count < 75 000	16 (17.4)
	Bilirubin > 3.2 mg/dL	25 (27.2)
	Albumin < 30 g/L	46 (50)
	Mean MELD score	9.43 ± 7.58
Tumor characteristics	Single nodule	41 (44.6)
	2-3 nodules	19 (20.7)
	Diffuse	33 (35.9)
	Portal vein obstruction[1]	31 (34.8)
Follow-up and survival	HCC as first presentation of liver disease	53 (57.6)
	Mean F/U prior to HCC in those with known liver disease	40.2 mo
	Mean follow-up after HCC diagnosis	16.4 mo
	Mean survival	14.9 mo

[1]Three patients had multiple nodules without details regarding vascular invasion or size of the nodules.

Tumors more than 5 cm in diameter were considered eligible for surgical resection when there was no evidence of vascular invasion and when surgical resection was technically possible. These patients were not considered eligible for any other curative treatment namely liver transplantation, percutaneous ethanol ablation, or radio frequency ablation. Patients who had tumors eligible for a curative treatment were further selected according to transplantation indication with an upper age limit of 65 years.

Statistical analysis

Biochemical data were collected at the time of diagnosis of hepatocellular carcinoma. Quantitative data were expressed as mean ± standard deviation and range. Group comparisons used the two-sample *t*-test or the Mann-Whitney test for quantitative variables and the two-tailed Fischer's exact test was used for qualitative variables. Comparison of survival used the Kaplan-Meier model, the log-rank test and receiver operating characteristic (ROC) curve.

RESULTS

Etiology

Characteristics of the 92 HCC patients are shown in Table 1. The mean age was 60.5 ± 22.3 years and the sex ratio was 5.6:1 (M/F, 78/14). In 77 patients (83.7%), the etiology of the underlying liver disease was viral. HBV infection was found in 62 patients (67.4%) compared to 18 patients (19.6%) with HCV infection. Alcohol abuse was found in 22 patients (23.9%) but was the only cause of

liver disease in only 8.2% of our patients. Miscellaneous causes included autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and tyrosinemia and accounted for 5.9% of cases.

Liver disease severity

HCC occurred in the setting of cirrhosis in all but one patient. The Child-Pugh class at time of diagnosis of HCC was A, B, and C in 32 (34.8%), 35 (39.3%), and 23 (25.8%) cases respectively. Model of end stage liver disease (MELD) score was calculated in 79 patients, and the mean was 9.4 ± 7.6. MELD score was < 12, 12-16, 16-24, and >24 in 68.4%, 12.7%, 12.7%, and 6.3% of patients respectively.

Tumor characteristics

The diagnosis of HCC was established during follow-up of liver cirrhosis in 36 patients (42.4%) with a mean follow-up period of 40 mo (median = 24 mo). In 13 patients (36.1%), HCC was diagnosed during the first two years following the diagnosis of cirrhosis, and in 26 patients (72.4%), within 5 years. The size of tumor nodules ranged from 1 cm to 20 cm with a mean of 5.6 cm. The number of nodules was 1, 2, and 3 in 41 (46.1%), 14 (15.7%), and 5 (5.6%) patients respectively; 29 patients (32.6%) had diffuse HCC. Portal vein thrombosis was present in 29 patients (38.2%). Forty patients (43.5%) had a single tumor of less than 5 cm or less than 3 nodules < 3 cm of diameter; ten of those patients had evidence of vascular invasion. The number of patients having a tumor considered eligible for a curative treatment was 32 (34.8%) versus 60 (65.2%) that were ineligible for curative resection on account of diffuse disease or tumor-associated with portal vein thrombosis.

Eligibility for curative treatment

Thirty-two patients had tumors considered eligible for curative treatment, but only 15 (46.9%) patients could benefit from such therapy. This was primarily due to the inability to perform a curative treatment at first intent because of age, tumor location for surgical resection, the lack of regular organ allocation program, or general contraindications other than hepatic disease severity quantified by the Child-Pugh score. When patients older than 70 or 65 were excluded, eligibility for a curative treatment decreased to 25.8% or 20.2% respectively. Other causes of failure to treat were the surveillance of small nodules of 1-2 cm of diameter, or patients awaiting OLT with no other therapeutic possibilities. Fifty-nine patients (64.1%) did not benefit from any treatment because of either decompensated liver disease or advanced tumor cases; in the remaining cases, treatment consisted of percutaneous ethanol injection (7 patients), chemoembolization (17 patients), OLT (8 patients) and surgical resection (7 patients). Eight patients had more than one treatment modality. There was a trend towards a difference in eligibility for treatment between patients presenting with HCC as first manifestation of liver disease (21% eligible for a curative treatment), compared to those discovered on routine screening for HCC (41% eligible for a curative treatment), although this difference did not reach statistical significance ($P = 0.13$).

Table 2 Univariate analysis of overall survival in HCC patients

	Criteria	Median survival (mo)	Mean survival (mo)	Log rank P
Age at diagnosis of HCC (yr)	< 55	18	40	0.008
	> 55	8	14	
Child-Pugh score	A	25	31.44	0.103
	B	10	18.14	
	C	4	24.5	
Bilirubin	< 3.2 mg/dL	15	23	0.015
	> 3.2 mg/dL	3	14	
HCC diagnosis	Known liver disease	25	33	0.003
	As first manifestation of liver disease	6	12	
Eligibility for treatment	No	15	6	0.005
	Yes	27	33	
INR	< 2	12	23	0.029
	> 2	2	9	
Portal vein thrombosis	Absent	14	23	0.021
	Present	3	12	

Survival and follow-up

Overall survival was 44.8%, 32.8%, and 17.6% at 1, 2, and 3 years respectively. Individual prognostic factors were studied using the Log rank method. Survival was studied with respect to multiple parameters including prothrombin time, platelet count, bilirubin value, albumin, creatinine, alpha-fetoprotein, the diameter of the largest nodule, the presence of portal vein thrombosis, eligibility for curative treatment, Child-Pugh score, MELD score, the presence of ascites, the etiology of liver disease, and the grading of esophageal varices. Prognostic factors identified in univariate analysis included age > 55, bilirubin < 3.2 mg/dL, HCC as the first manifestation of liver disease, eligibility for a curative treatment, International Normalized Ratio (INR) < 2, MELD score > 18, and the presence of portal vein thrombosis. Univariate analysis of overall survival (Table 2) failed to demonstrate a significant difference in survival with respect to Child-Pugh score in HCC patients. The mean survival was 31.4, 18.1, and 24.4 months respectively in Child-Pugh class A, B, and C patients ($P = 0.10$). In multivariate analysis using Cox regression model; survival in HCC patients was related to age < 55 at diagnosis of HCC ($P = 0.004$), bilirubin value < 3.2 mg/dL ($P = 0.014$), and HCC developing in the setting of known liver disease ($P = 0.016$).

Factors associated with six month survival are shown in Table 3. Death occurred within six months in 31 patients (38.3%). Survival beyond six months was associated with INR < 2, bilirubin < 3.2 mg/dL, tumor less than 5 cm of diameter or three nodules of less than 3 cm of diameter without portal vein thrombosis, MELD score < 16, lower Child-Pugh class, and the absence of portal vein thrombosis. Mean creatinine level, INR, and MELD score were lower in patients with > 6 mo survival. In multivariate analysis using Cox regression model, bilirubin

Table 3 Factors associated with six months survival *n* (%)

	Survival[1]		
	< 6 mo	> 6 mo	*P*
Child-Pugh score A	4 (14.3)	14 (35.9)	0.032[2]
INR < 2	22 (70.9)	43 (93.5)	0.012[2]
Bilirubin < 3.2 mg/dL	17 (58.6)	35 (83.3)	0.033[2]
MELD score < 16	37 (90.2)	18 (62.1)	0.006[2]
Portal vein thrombosis	17 (54.8)	13 (30.9)	0.035[2]
Tumor < 5 cm or 3 nodules < 3 cm without portal vein thrombosis	3 (10.3)	11 (31.4)	0.042[2]
Mean creatinine (mg/dL)	1.57 ± 0.89	1.19 ± 0.49	0.021[3]
Mean INR	1.68 ± 0.47	1.47 ± 0.42	0.041[3]
Mean MELD score	13.26 ± 8.12	8.12 ± 6.54	0.005[3]

[1]Patients treated curatively by OLT or surgical resection were excluded from this analysis. [2]Fischer exact test. [3]Mean comparisons by independent sample *t*-test.

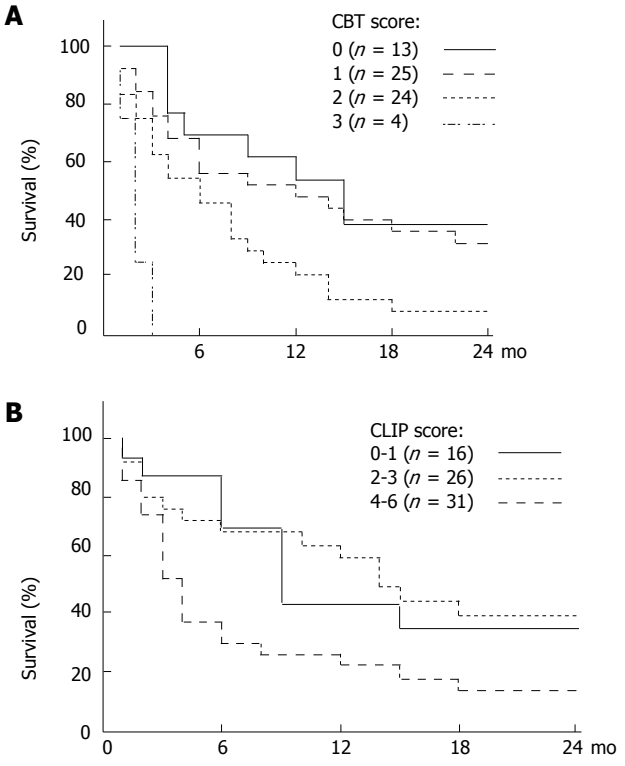


Figure 1 **A**: Kaplan-Meier survival curves for 68 patients with hepatocellular carcinoma based on 3 clinical criteria CBT (Creatinine, Bilirubin, and Tumor as primary manifestation); **B**: Kaplan-Meier estimated survival curves according to the Cancer of the Liver Italian Programme (CLIP) classification.

> 3.2 mg/dL ($P = 0.001$), creatinine > 1 mg/dL ($P = 0.017$), and HCC as the first manifestation of liver disease ($P = 0.035$) were independent predictors of survival less than six months ($P = 0.006$). Short-term survival (> 6 mo) was 69.2%, 56%, 45.8%, and 0% when none, one, two, or all of these 3 independent factors were present ($P = 0.0002$) (Figure 1). In comparison, the CLIP score predicted short-term survival in our patient population was 69.3%, 68%, and 29.6% when the score was respectively 0-1, 2-3, or 4-6 (Log rank $P = 0.047$) (Figure 1). A score based on the 3 clinical variables identified above (CBT score for creatinine, bilirubin, and tumor) outperformed the CLIP score by Cox proportional hazard. ROC curve showed

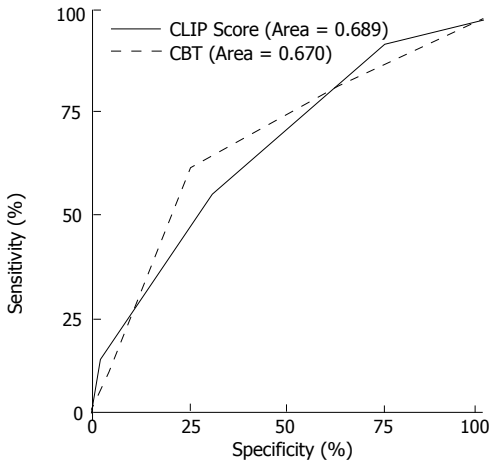


Figure 2 Receiver Operating Characteristic (ROC) curve for early mortality using the CBT (Creatinine, Bilirubin, and Tumor as primary manifestation) and CLIP scores.

both prognostic models to be equivalent and of moderate accuracy (Figure 2).

DISCUSSION

HCC is a relatively rare cancer in Lebanon, ranking 14th among both males and females, with an age standardized rate of 3.5 and 2.2 per 100 000 respectively[11]. Understanding the risk factors for HCC and predictors of poor survival are important in prevention and treatment. The results of our study show that most HCC patients in Lebanon have HBV-related liver cirrhosis, accounting for nearly two thirds of our patients. This is concordant with previous findings regarding the etiology of liver cirrhosis in Lebanon where HBV was the most important cause accounting for more than 50% of cirrhosis, followed by HCV, and alcohol abuse[12]. Of note, the HBV and HCV carrier state in Lebanese blood donors is 1%-2% and 0.1%-0.6% respectively[13-15]. Interestingly, there were no cases of non-alcoholic steatohepatitis- or cryptogenic cirrhosis-related HCC in this study population. Studies from neighboring Middle Eastern countries showed wide variation in the etiology of HCC. In Saudi Arabia, HCV plays a major role in the epidemiology of HCC where 39.5% of patients were anti-HCV antibody positive[8]. This is in contrast to an overall HCV seroprevalence of 1.1%-2.9% among Saudi- blood donors[9] versus 0.6% among Lebanese-blood donors[13]. In Jordan, an association between HDV-positive status and HBs-antigen positive primary HCC was found. The prevalence of hepatitis D viral infection in Jordan was 23% in patients with chronic liver disease and in 67% of patients with HCC[7]. In contrast, none of our HCC patients was seropositive for HDV. Lastly, HBV infection was found to be the leading cause of HCC in Turkey, followed by HCV infection, and alcoholic liver disease[6]. Hepatitis B, hepatitis C, excessive alcohol intake were detected in 56%, 23.2%, and 15.9% of Turkish HCC patients respectively.

Previous studies have identified multiple prognostic factors in HCC[16-19]. Gender differences were studied showing significantly longer median survival in females (14 mo) than in male patients (4 mo)[20]. This does not

seem to be the case in our patients. Previous multivariate analysis demonstrated that high serum alpha-fetoprotein levels, venous invasion, extrahepatic metastasis, and lack of therapy were independent factors related to unfavorable prognosis[17]. In another study, only type of estrogen receptors and bilirubin showed independent predictive value for mortality[21]. In Child B and C cirrhosis patients, factors predictive of prolonged survival were albumin level (≥ 30 g/L), absence of esophageal varices, tumor size (≤ 3.0cm), tumor number (solitary), and alpha-fetoprotein (AFP) level (< 400 ng/mL)[18]. In our study, factors predictive of prolonged survival were bilirubin (< 3.2 mg/dL) at diagnosis, age (less than 55), and HCC developing in the setting of known liver disease. Serum creatinine and INR values were not independent factors associated with long-term survival, nor was the etiology of the underlying liver disease. This explains somehow that the MELD score was associated with the outcome in univariate analysis, whereas this score has limited value for HCC since prognosis is not only related to liver failure, but also to tumor evolution which is not taken into account in this score. Furthermore, the etiology of underlying liver disease does not seem to play a role in prognosis either in univariate or multivariate analysis. The finding that HCC developing in the setting of known liver disease is an independent predictor of outcome further supports the role of routine screening for HCC in cirrhosis since some of those patients may be amenable to curative treatment.

Data on short-term survival, particularly from the Middle East, is scarce in the literature. Such data is important for the evaluation of early mortality on liver transplantation waiting lists. In a study from Kuwait involving 145 HCC cases, the CLIP score was found to be superior to the Okuda stage as a prognostic indicator[22]. However, the results from a study by Siddique et al seem to reflect the natural history of the disease (91% did not receive any specific therapy for HCC)[22]. In our study, factors predictive of six month survival were bilirubin, creatinine, and HCC developing in the setting of known liver disease. Even for short-term survival, MELD score does not seem to be fit for the evaluation of early mortality in HCC patients. MELD score did, however, show a significant difference in Kaplan-Meier survival analysis when the chosen cut-off was 16, but no difference in survival was found when the cut-off was 24. Again, the etiology of liver disease does not seem to play a role in the early mortality of HCC neither in univariate nor in multivariate analysis.

Our study is limited by the fact that the number of patients was relatively small which restricts the number of variables that can be examined with confidence by multivariate analysis. HCC is however, an uncommon cancer in Lebanon, ranking 14th among both males and females, with an age standardized rate of 3.5 and 2.2 per 100 000 respectively[11]. With a population of just over 3 million, the number of HCC in the whole of Lebanon over a one-year period is estimated at less than 90 cases. It is estimated that a third to half of these cases are referred to the three major university hospitals involved in this study for evaluation and management. We believe, therefore, that our study cohort is relatively large for Lebanon and is likely to be well representative of the population at large particularly as it relates to etiology and outcome.

In conclusion, our study shows that HCC in Lebanon is primarily related to HBV cirrhosis with HCV and alcohol abuse ranking as distant second and third risk factors. The probability for a curative treatment is still low in Lebanon because of advanced tumor stage at presentation and the lack of a regular liver donation program. Thus determination of independent prognostic factors for early mortality is necessary, which include bilirubin (> 3.2 mg/dL), creatinine (> 1 mg/dL), and HCC as first presentation of liver disease. Further studies are needed to validate the above identified prognostic factors and to evaluate drop-out and mortality on the waiting list taking into account these factors.

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Apoptosis of human colon carcinoma HT-29 cells induced by ceramide

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Abstract

AIM: To investigate the effect of exogenous ceramide-induced apoptosis on human colon carcinoma HT-29 cells.

METHODS: Light microscope, transmission electron microscope and fluorescence microscope were used to observe the morphology change of apoptosis in HT-29 cells. Agarose gel electrophoresis was performed to detect the DNA fragment. Mitochondrial function was detected by MTT assay. mRNA expression of Bcl-2 family gene members was determined by reverse transcription polymerase chain reaction (RT-PCR) assay.

RESULTS: After C_2-ceramide treatment, typical characteristics of apoptosis, such as nuclear chromatin breakage, apoptotic body and DNA ladder, could be observed. After exposure to 50 μmol/L C_2-ceramide for 12 and 24 h, cell apoptosis was 64.1% and 81.3% respectively, which had a time-and dose-effect relationship. Mitochondrial function started to decrease from 6 h after exposure to ceramide. Meanwhile, ceramide up-regulated or down-regulated the mRNA expression of Bcl-2 family gene members.

CONCLUSION: Ceramide induces apoptosis of human colon carcinoma HT-29 cells by affecting the expression of Bcl-2 family gene members and impacting the mitochondrial function.

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Key words: Ceramide; Apoptosis; Human colon carcinoma cells; Bcl-2 family gene member; Mitochondrial function

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INTRODUCTION

Ceramide (N-acetyl-sphingosine) is a fundamental unit of sphingomyelin which is a moiety of cytoplasmic membrane. Ceramide, also an important bioactive compound *in vivo*, can conduct specific signal transduction from cell surface receptor and environmental stress to nuclei, and then provoke multiple cytobiologic effects[1]. Meanwhile, ceramide is a common second messenger molecule participating in sphingomyelin cycle, apoptosis and differentiation of many cell types[2,3]. Apoptosis can be induced by surrounding stimulating factors, such as tumor necrosis factor α (TNF-α), endotoxin, FAS ligand, radiation, chemotherapeutics and heat shock, and is associated with ceramide. Recent studies indicate that ceramide has a close relationship with genesis and progression of digestive tract tumor. But little information about Bcl-2 family gene member and mitochondrial function is available concerning the effect of ceramide-induced apoptosis in colon carcinoma which is one of the most aggressive forms of cancer. Even ceramide could induce apoptosis of HT-29 cells. This study was to determine how ceramide induces apoptosis of human colon carcinoma cells and to discuss its mechanism.

MATERIALS AND METHODS

Materials

C_2-ceramide (N-acetyl-D-sphingosine) was purchased from Sigma, dissolved to 5 mmol/L in DMSO as a stock solution. Hoechest 33 258 and MTT were purchased from Sigma, dissolved in PBS solution. The two solutions were preserved at 4℃ from light. Apoptosis DNA extract kit was from Shanghai Huashun Biotechnology Company. RPMI-1640 media, DMSO and fetal bovine serum (FBS) were bought from GIBCO. Trypsin and EDTA were obtained from Amresco.

Cell culture

HT-29 cells were provided by the Institute of Tumor Prevention and Cure in Beijing, China. Cells were cultured in RPMI-1640 media supplemented with 10%

Table 1 Primer sequences of Bcl-2 family gene members

Gene	Primer sequence	Size (bp)	Temperature (℃)
Bax	Sense: 5′-CCAGCTGCCTTGGACTGT-3′ Antisense: 5′-ACCCCCTCAAGACCACTCTT-3′	135	61
Bcl-xl	Sense: 5′-GTAAACTGGGGTCGCATTGT-3′ Antisense: 5′-TGGATCCAAGGCTCTAGGTG-3′	146	60
Bcl-2	Sense: 5′-GGCAATGTGACTTTTTCCAA-3′ Antisense: 5′-GGCTGATATTCTGCAACACTG-3′	137	55
Bad	Sense: 5′-AGGGCTGACCCAGATTCC-3′ Antisense: 5′-GTGACGCAACGGTTAAACCT-3′	178	60
Bid	Sense: 5′-GCTTCCAGTGTAGACGGAGC-3′ Antisense: 5′-GTGCAGATTCATGTGTGGATG-3′	116	60
β-actin	Sense: 5′-GTGGACATCCGCAAAGAC-3′ Antisense: 5′-TCAACGCAATGTGGGAAG-3′	303	58

FBS and 2 mmol/L glutamine. Antibiotics added to the media were 100 U/mL penicillin and 100 U/mL streptomycin. Cells maintained in a humidified atmosphere of 95 mL/L air and 50 mL/L CO_2 at 37℃ were then passed at pre-confluent densities in a solution containing 0.25% trypsin and 0.02% EDTA. Exponentially growing cells were used throughout all experiments.

Light microscopy

HT-29 cells were treated with C_2-ceramide at final concentrations of 12.5, 25 and 50 μmol/L for 24 and 48 h respectively. DMSO served as negative control. During the procedure, cell morphology was observed under light microscope for different time.

Transmission electron microscopy

Cells were collected and fixed with 4% glutaraldehyde in phosphate buffer overnight at 4℃. After post-fixation with 1% OsO_4 in cacodylate buffer for 1h at 4℃, the pellet was dehydrated in graded ethanol solutions and embedded in Epon. Ultrathin sections of pellet were counterstained with uranyl acetate and lead citrate and observed under transmission electron microscope.

Fluorescence microscopy

Cells (4×10^4) were treated as above, suspended with FBS and incubated with Hoechest 33 258 for 30 min at 37℃ from light. Then the cells were observed under fluorescence microscope. Three hundred cells were counted and apoptotic cell rate was calculated.

Detection of DNA fragmentation

Cells (2×10^6) were treated as above and collected. Fragmented DNA was extracted according to the manufacturer's instructions of apoptosis DNA extract kit and underwent electrophoresis on 2 g/L agarose gel containing 0.5 g/L of ethidium bromide and visulized by UV transillumination.

Detection of mitochondrial function (MTT assay)

Cells (1.5×10^4) were cultured in 96-well plates and treated with C_2-ceramide for 1, 3, 6, 9, 12 and 24 h as above. Then MTT and DMSO were added by turns. Absorbance was determined with a multi-well plate reader at wavelength 570 nm.

Table 2 Apoptosis rate of HT-29 cells induced by C_2-ceramide (mean ± SD, %)

C_2-ceramide	Cell count (*n*)	12 h	24 h
0 μmol/L	300	5.33 ± 1.53	8.67 ± 2.52
12.5 μmol/L	300	14.7 ± 2.52[a]	28.0 ± 9.85[a]
25 μmol/L	300	33.3 ± 8.37[a]	43.3 ± 12.3[a]
50 μmol/L	300	64.7 ± 7.51[a]	81.3 ± 9.07[a]

[a]$P < 0.05$ *vs* control.

RT-PCR

Cells (1×10^6) were treated for 24 h as above. Total RNA was isolated from the treated cells with Trizol and reversely transcribed into cDNA with human specific primers for Bax, Bad, Bid, Bcl-2, Bcl-xl and β-actin. Sequences of primers are shown in Table 1. Briefly, 35 cycles of PCR amplification were performed at 94℃ for 30 s, annealing at 60℃ for 30 s and extension at 72℃ for 30 s in a 25 μL reaction system. PCR products were confirmed by 1.5 g/L agarose gel electrophoresis and visualized by UV transillumination. mRNA expression of genes was assessed by correcting housekeeping gene β-actin, which served as an internal control.

Statistical analysis

All statistical analyses were performed with SPSS 10.0 statistical package for Microsoft Windows. Data were expressed as mean ± SD for all measurements. $P < 0.05$ was considered statistically significant.

RESULTS

C_2-ceramide induced morphology changes of apoptosis in HT-29 cells

With increasing concentration of C_2-ceramide and exposure time, HT-29 cells became round, atrophic and poorly adherent and floating cells increased under light microscope. Cells from control showed normal distribution and morphology in all cellular organelles except for a few necrotic cells under transmission electron microscope. The main ultra-microstructural changes seen in all treated groups were chromatin aggregation, mitochondrial denaturation and apoptotic body, as well as cytoplasmic compartments, swelling and disappearance of mitochondrial cristae, *etc* (Figure 1). The necrotic changes were more pronounced in cells treated with 50 μmol/L C_2-ceramide.

Apoptotic cells determined by Hoechest 33 258 assay increased in a time- and dose-dependent manner after treatment with C_2-ceramide (Table 2). Normal cellular chromatin did not change and uniformly spread over the whole nuclei displaying diffusion uniformity fluorescence. However, apoptotic chromatin was identifiable by its scattered drop-like structure locating on the area of the original nuclei which displayed high brightness lump or punctiform fluorescence. The total size of apoptotic nuclei

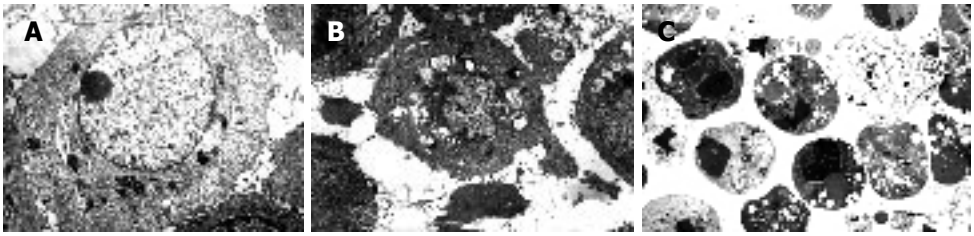


Figure 1 Morphology change of apoptosis in HT-29 cells induced by C_2-ceramide at different concentrations for different time under transmission electron microscope. **A**: 0 μmol/L, 24 h, × 5000; **B**: 50 μmol/L, 24 h, × 4000; **C**: 50 μmol/L, 48 h, × 3000.

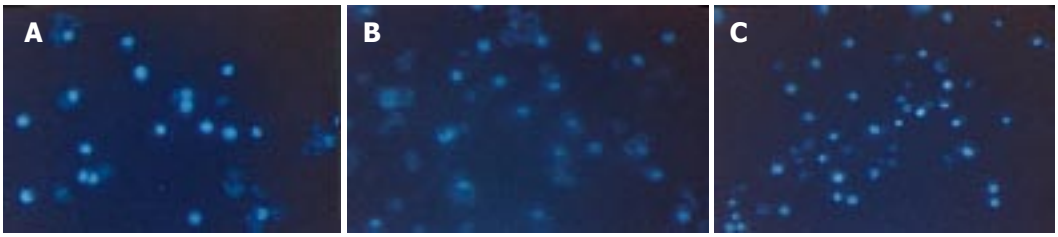


Figure 2 Morphology change of apoptosis in HT-29 cells induced by C_2-ceramide at different concentrations for different time under fluorescence microscope. **A**: 0 μmol/L, 24 h, × 100; **B**: 50 μmol/L, 12 h, × 100; **C**: 50 μmol/L, 24 h, × 100).

Table 3 C_2-ceramide damaged mitochondria function of HT-29 cells (mean ± SD)

C_2-ceramide (μmol/L)	1 h	3 h	6 h	9 h	12 h	24 h
0	0.505 ± 0.061	0.596 ± 0.050	0.528 ± 0.083	1.088 ± 0.134	1.371 ± 0.066	1.770 ± 0.161
12.5	0.478 ± 0.044	0.609 ± 0.048	0.502 ± 0.029	0.833 ± 0.149[a]	1.036 ± 0.122	1.560 ± 0.220
25	0.487 ± 0.051	0.568 ± 0.048	0.404 ± 0.033[a]	0.646 ± 0.158[a]	0.760 ± 0.086[a]	1.484 ± 0.346[a]
50	0.490 ± 0.054	0.541 ± 0.049	0.345 ± 0.050[a]	0.456 ± 0.071[a]	0.708 ± 0.073[a]	1.342 ± 0.061[a]

[a]$P < 0.05$ *vs* control.

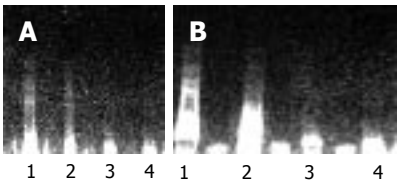


Figure 3 DNA fragmentation induced by C_2-ceramide in HT-29 cells for 12 h (**A**) and 24 h (**B**). 1: 50 μmol/L; 2: 25 μmol/L; 3: 12.5 μmol/L; 4: 0 μmol/L.

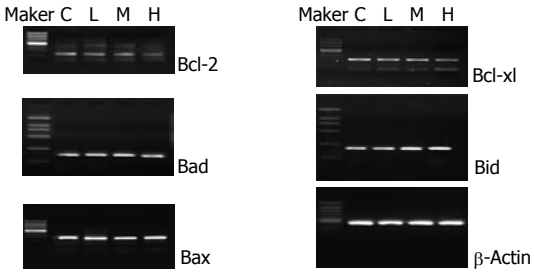


Figure 4 Expression of apoptosis-related genes in HT-29 cells induced by C_2-ceramide for 24 h (C: 0 μmol/L; L: 12.5 μmol/L; M: 25 μmol/L; H: 50 μmol/L).

appeared smaller and more shrunken than the intact cells (Figure 2).

C_2-ceramide induced DNA fragmentation of HT-29 cells

DNA ladder was seen through DNA agarose gel electrophoresis, especially pronounced in the cells treated with 50 and 25 μmol/L C_2-ceramide for 12 and 24 h. Cells treated with 12.5 μmol/L C_2-ceramide and DMSO kept their integrity, having no ladder appearance (Figure 3).

C_2-ceramide damaged mitochondrial function of HT-29 cells

The absorbance value increased gradually with increased concentration of C_2-ceramide and prolongation of exposure time. But the absorbance value after treatment with 50 and 25 μmol/L C_2-ceramide was obviously lower than that in the control, showing statistical significance from 6 to 24 h, indicating that C_2-ceramide could decrease mitochondrial function (Table 3).

C_2-ceramide affected mRNA expression of apoptosis-related genes

After cells were treated with C_2-ceramide for 24 h, the mRNA expressions of Bax, Bad and Bid genes were up-regulated. However, the expressions of Bcl-2 and Bcl-xl were down-regulated (Figure 4). Moreover, the ratio of Bcl-2 to Bax in 50 μmol/L C_2-ceramid group was less than 1.

DISCUSSION

Recently, the importance of ceramide in cell metabolism has been broadly investigated. The biological effect of ceramide on different cell lines is different. The role of ceramide-induced apoptosis has been confirmed[4]. Recent studies indicate that ceramide is a common second

messenger molecule of apoptosis. Apoptosis induced by stimulating factors is mediated by ceramide from sphingomyelin circulation way[5,6]. The effect of ceramide on apoptosis has been studied extensively in neoplastic cells but rarely in solid tumor cells. Ceramide is closely related with genesis and progression of digestive tract tumor. Decreased ceramide content increases the risk of developing digestive tract tumor since ceramide content in human colon carcinoma cells is lower than that in normal colon mucosa cells[7].

To some extent, tumor chemotherapy is to induce apoptosis. In this experiment, human colon cancer cells were exposed to exogenous C_2-ceramide. Results indicated that C_2-ceramide could induce typical characteristics of apoptosis, such as nuclear chromatin break and apoptotic body as well as DNA ladder in a time-and dose-dependent manner. Succinate dehydrogenase (SDH) in the mitochondria is an index of cellular respiration and energy, which reflects mitochondrial function. MTT assay suggested that C_2-ceramide could decrease mitochondrial function. By studying Bcl-2 family gene members, we also found that C_2-ceramide could up-regulate or down-regulate the mRNA expression of these genes, suggesting that exogenous C_2-ceramide induces apoptosis of human colon carcinoma cells *in vitro* by affecting the expression of Bcl-2 family gene members and damaging the mitochondrial functions.

C_2-, C_6 and C_8-ceramides could induce apoptosis of cell lines, but dihydroxy-ceramide lacking C_4-C_5 trans-double bond located in basal framework of sphingolipid could not induce apoptosis[8].

Studies also indicate that ceramide has many target sites inducing apoptosis, such as ceramide-activated protein kinase (CAPK)[9], ceramide-activated protein phosphatase (CAPP)[10], protein kinase C (PKC) family[11], stress-activated protein kinase/c-JUN N-terminal kinase (SAPK/JNK)[12] and caspase cascade reaction[13]. There is a body of evidence that mitochondria are involved in apoptosis, for example, release of cytochrome C from mitochondria triggers apoptosis[14-16].

In conclusion, mitochondria is the target of ceramide. The importance and mechanism of mitochondria in ceramide-induced apoptosis need to be further studied.

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BASIC RESEARCH

Gene expression profiling defined pathways correlated with fibroblast cell proliferation induced by *Opisthorchis viverrini* excretory/secretory product

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Abstract

AIM: To investigate the mechanism of fibroblast cell proliferation stimulated by the *Opisthorchis viverrini* excretory/secretory (ES) product.

METHODS: NIH-3T3, mouse fibroblast cells were treated with *O. viverrini* ES product by non-contact co-cultured with the adult parasites. Total RNA from NIH-3T3 treated and untreated with *O. viverrini* was extracted, reverse transcribed and hybridized with the mouse 15K complementary DNA (cDNA) array. The result was analyzed by ArrayVision version 5 and GeneSpring version 5 softwares. After normalization, the ratios of gene expression of parasite treated to untreated NIH-3T3 cells of 2-and more-fold upregulated was defined as the differentially expressed genes. The expression levels of the signal transduction genes were validated by semi-quantitative SYBR-based real-time RT-PCR.

RESULTS: Among a total of 15 000 genes/ESTs, 239 genes with established cell proliferation-related function were 2 fold-and more-up-regulated by *O. viverrini* ES product compared to those in cells without exposure to the parasitic product. These genes were classified into groups including energy and metabolism, signal transduction, protein synthesis and translation, matrix and structural protein, transcription control, cell cycle and DNA replication. Moreover, the expressions of serine-threonine kinase receptor, receptor tyrosine kinase and collagen production-related genes were up-regulated by *O. viverrini* ES product. The expression level of signal transduction genes; *pk*C, *pdgfrα, jak* 1*, eps* 8*, tgfβ* 1*i*4, *strap* and *h ras* measured by real-time RT-PCR confirmed their expression levels to those obtained from cDNA array. However, only the up-regulated expression of *pkC, eps* 8 *and tgfβ* 1*i*4 which are the downstream signaling molecules of either epidermal growth factor (EGF) or transforming growth factor-β (TGF-β) showed statistical significance ($P < 0.05$).

CONCLUSION: *O. viverrini* ES product stimulates the significant changes of gene expression in several functional categories and these mainly include transcripts related to cell proliferation. The TGF-β and EGF signal transduction pathways are indicated as the possible pathways of *O. viverrini*-driven cell proliferation.

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Key words: Gene expression profile; *Opisthorchis viverrini*; Excretory/secretory product; cDNA array; Fibroblast; Cell proliferation; Signal transduction; Cholangiocarcinogenesis

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INTRODUCTION

An *Opisthorchis viverrini* infection or opisthorchiasis is the most important health problem in Southern Asia, including Northeastern Thailand, Loas, Vietnam and southern China[1]. It is a definite cause of bile duct cancer or cholangiocarcinoma (CC) in humans and has been classified as a carcinogen. Parkin *et al*[2] performed

a case-control study of patients and estimated that two-thirds of CC cases in Thailand were caused by *O. viverrini* infestation. Nowadays, CC remains a major public health problem in many parts of Southeast Asia. The incidence rate of CC around the world is different with increasing tendency[3,4]. The highest incidence is in the Northeastern part of Thailand with the rate of 188 per 100 000[5]. The pathogenesis of *O. viverrini*-associated CC may be a complex process, involving several mechanisms. The effect of parasites on the bile duct epithelium is classified, as both mechanical and chemical irritations. The mechanical irritation is the direct contact of parasites to the bile duct epithelium; the chemical irritation is believed to be caused by the excretory/secretory (ES) product released from the flukes. These chronic irritations caused by the parasite later result in the proliferation of bile duct epithelium and leads to epithelial hyperplasia[6]. This is an important step in the genesis of cancer because hyperplastic cells are vulnerable to a carcinogen and can easily turn to adenomatous and finally cancerous cells[7]. Moreover, the alterations of immune response and the stromal cells which are mainly composed of fibroblasts were observed in opisthorchiasis and CC in both animal models and human. The fibrosis and mononuclear cell infiltration with lymphocyte aggregation and, additionally, ductal dilatation were observed in the gall bladders and extrahepatic bile ducts of the hamsters infected with *O. viverrini*[8]. In addition, the chronic inflammation and fibrosis of the bile ducts may contribute to the strikingly enhanced susceptibility to CC among people with heavy liver fluke infection[9]. The malignant transformation of bile duct epithelium *via* dysplasia was detected in the abnormal intrahepatic biliary tree of the patients with congenital hepatic fibrosis[10]. There is the important relationship between the gradual decreases of inflammation with a concomitant increase in fibrosis after *O. viverrini* re-infection in the experiment hamsters[11]. All of these data provide evidence of the importance of fibrosis in the formation of cholangiocarcinoma.

Fibrosis is the excessive accumulation of extracellular matrix proteins produced from the active and accumulated fibroblasts. It occurs in most types of chronic diseases including chronic liver disease. Liver fibrosis is considered a model of the wound-healing response of the liver to repeated injury[12]. Activated hepatic stellate cells, portal fibroblasts, and myofibroblasts have been identified as the major collagen-producing cells in the repeatedly injured liver[13]. In the fibrogenesis, the increased numbers and then the accumulation of fibrogenic cells including the fibroblasts and myofibroblasts are the main observations. As aforementioned, there is the correlation of fibrosis with the development of CC. The interaction of *O. viverrini* and the host immune cells has been proven to be the main causative issue for chronic inflammation in opisthorchiasis. The cytokines released from the immune cells will lead to the formation of fibrosis later. Moreover, direct contact of *O. viverrini* which have been strongly proven to induce bile duct epithelium erosion and cell proliferation replacement that occurs later on, may indirectly induce the surrounding fibroblasts around the affected area to proliferate[14]. The importance of hyperproliferative fibroblasts regarding the induction of epithelial cancer has been increasingly reported[15,16]. Though this phenomenon has not been investigated in CC, the direct effect of *O. viverrini* ES product to induce fibroblast cell proliferation has recently been reported *in vitro*[14,17]. So far, there are no data for the explanation of the fibroblast cell proliferation induced by the *O. viverrini* ES product.

From all the evidence described above, it would appear to be of great interest to study the mechanism of how *O. viverrini* ES product induces fibroblast cell proliferation. In order to know the response of cell to *O. viverrini* ES product, the gene expression analysis of fibroblast cell non-contact co-cultured with adult *O. viverrini* was performed. The expression level of genes was compared to those without *O. viverrini* ES product treatment. The cell proliferation-associated gene expression was discussed in relation to their roles in *O. viverrini* ES product-induced fibroblast cell proliferation. The expression levels of some signal transduction genes were also analyzed and validated by real-time RT-PCR to indicate the possible signal transduction pathway(s) utilized by *O. viverrini* in the induction of fibroblast cell proliferation.

MATERIALS AND METHODS

Parasite preparation

Opisthorchis metacercariae were obtained from naturally infected cyprinoid fish captured from fresh-water reservoirs in the endemic area of Khon Kaen province, Thailand. Pepsin-HCl was used to digest the flesh to obtain metacercariae, which were then introduced to the 6-8-wk-old hamsters *ad libitum*. After 1 mo, the adult *O. viverrini* were collected from the bile ducts of the hamsters and were washed several times with PBS containing 100 mg/L penicillin and 100 kU/L streptomycin. To prevent microbial contamination, the adult *O. viverrini* were then incubated for 30 min in PBS containing antibiotics before being used in the non-contact co-culture technique. The animal holding protocol was approved by the Animal Ethics Committee of Khon Kaen University (AEKKU011/04), based on the Ethics of Animal Experimentation of National Research Council of Thailand.

Cell culture and non-contact co-culture

To prepare the fibroblasts stimulated with *O. viverrini* ES product, the non-contact co-culture between the fibroblasts and the adult parasites was performed. The mouse fibroblast cell line, NIH-3T3, was bought from the American Type Culture Collection (ATCC; Manassas, VA, USA). It was maintained in DMEM (Gibco, Gaithersburg, MD, USA) containing 100 mL/L calf serum (CS) (Gibco, Gaithersburg, MD, USA), designated as the complete medium, followed by incubation in a 50 mL/L CO_2 incubator at 37℃ and passaging twice a week. Trypan blue staining was used to measure the viability of cells. The cells with more than 95% viability were collected for performing non-contact co-culture.

The NIH-3T3 cells and the intact viable *O. viverrini* were non-contact co-cultured in a 24-well transwell (Costar, Corning Incorporated, Cambridge, NY, USA). The

parasites were incubated in the upper chamber containing the 8-μm porous plate, which allowed the ES product to diffuse to the lower chamber where the NIH-3T3 cells adhered. The cell proliferation induction of ES product to the NIH-3T3 cells was confirmed by the viable cell count using trypan blue staining. Briefly, 10 000 NIH-3T3 cells were plated onto the lower chamber of a 24-well double-chamber culture plate for 2 d with complete medium to let most of cells adhere to the well. Then the media were changed to DMEM without CS, serum-free medium. Five viable adult *O. viverrini* were added to the upper chamber of each well. The NIH-3T3 cells cultured in complete medium and serum-free medium were used as positive and negative controls, respectively. The cell numbers obtained from the *O. viverrini* treatment cultured for 2 d was compared to those of the negative and positive controls with the same culturing time.

Hybridization and array data analysis

The in-house prepared mouse cDNA array was prepared by Dr. Kazuhiko Uchida, Department of Biochemistry and Molecular Oncology, Comprehensive Human Science, Graduate School, University of Tsukuba, Japan. It consists of 15 K of either mouse or human genes/ESTs. Firstly, the total RNA extraction from NIH-3T3 cells treated and untreated with adult *O. viverrini* ES product was performed. Briefly, total of 2-5 × 10^6 cells were collected in RNAlater solution (Ambion®, Hilden, Germany) and processed for total RNA with a commercial kit (ISOGEN; Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The RNA quality was confirmed by subjecting the samples to electrophoresis on formaldehyde-agarose gel and staining the 18S and 28S RNA bands for visualization. Then, a 2-μg portion of the intact total RNA was dissolved in 8 μL of RNase-free water, and 2 μL of oligo (dT) primer (1 g/L; Invitrogen, Carlsbad, CA, USA) was then added. The sample was heated for 10 min at 70℃ and immediately chilled on ice. The following reagents were then added to the sample: 5 μL of first-strand cDNA synthesis buffer, 1 μL of DTT (0.1 mol/L final concentration), 1.5 μL of deoxynucleotide triphosphates (20 mmol/L dATP, dGTP, and dTTP), 1.5 μL of SuperScript (200 MU/L; Invitrogen), and [α-^{33}P]dCTP (111 PBq/mol; Amersham). The reaction was incubated at 37℃ for 90 min. Labeled cDNA was then denatured for 3 min at 95℃. The array was prehybridized at 42℃ for 2 h in MicroHyb solution (Invitrogen, Carlsbad, CA, USA) with 0.5 mg/L poly(dA) and 1 mg/L *COT1* DNA (Invitrogen, Carlsbad, CA, USA). The probes were hybridized at 42℃ overnight in the same solution. The membrane array was washed twice at 50℃ with 2 × SSC-10 g/L SDS for 20 min and at room temperature with 0.5 × SSC-10 g/L SDS for 15 min. The membrane array was exposed to an imaging plate which was scanned with a BAS 5000 Imaging Analyzer (Fuji Film, Tokyo, Japan). Spot intensity was quantified with ArrayVision version 5.0 software (Imaging Research, Ontario, Canada). GeneSpring version 5.0 software (Silicon Genetics, Redwood, CA) was used to normalize values for each gene for data analysis.

Real-time RT-PCR

The mRNA was isolated using a Total RNA Extraction Miniprep System (Viogene, Fegersheim, France) according to the manufacturer's instructions. The DNA contaminated in the extracted RNA was destroyed by DNaseI (Promega Corporation, Madison, WI, USA) at 37℃ for 30 min. The DNaseI inactivation reaction was performed at 75℃ for 10 min. The first-strand cDNA was synthesized from 1 μg of mRNA using the Reverse Transcription System (Promega Corporation, Madison, WI, USA). Semi-quantitative PCR was performed with the SYBR-based method in 12.5 μL reaction in Rotor Gene RG-3000 (Corbett Research). After the denaturing of cDNA at 95℃ for 10 min, the reaction profile was subjected to 50 amplification cycles, each cycle consisted of denaturation at 95℃ for 30 s, annealing at different temperatures for each gene (52, 55, 58, 58, 54, 52, 52, 52, 65, and 60℃ for *abl* 1, *pk*C, *pdgfrα*, *jak* 1, *eps* 8, *tgfβ* 1*i*4, *strap*, *csnk* 1α1, *h ras, and* β_2*m*, respectively) for 30 s, and extension at 72℃ for 45 s. After the PCR, a melting curve was constructed by increasing the temperature from 50 to 99℃. β_2-microglobulin (β_2m), whose expression was found to be directly proportional to the amount of mRNA that presented in the sample stimulated by any growth factors[18], was used as the internal control. The standard curve between the CT of each gene expression and the amount of the starting total RNA was performed. The CT of the samples was then compared to the corresponding standard curve and quantitated the amount of the starting mRNA in that sample. The RT-PCR was performed three times for each gene.

Table 1 shows the PCR primers designed by the Gene Fisher Program according to the mRNA sequences of each gene in the GenBank database. The primer sequences can then be categorized into groups of growth factors they belong to, in this case, the signal transduction molecules. The result was analyzed for the statistical significance by STATA version 8 (StataCorp LP, Texas, USA).

RESULTS

Gene responding to O. viverrini ES product

Using a mouse cDNA array, we examined the gene expression profile of cell proliferation stimulated by *O. viverrini* ES product. A total of 15 000 genes and ESTs were tested. *O. viverrini* caused widespread alteration in gene expression. The ratios of gene expression between treated and untreated with the parasite ES product equal and greater than 2 were focused on because of their striking changes. Among all genes/ESTs in 15K cDNA array, 885 genes fitted to this criterion. Among these genes, only 536 genes had a variety of molecular functions and only 239 genes within these 536 genes had cell proliferation-related functions. These 239 genes were categorized in groups as proteins playing roles in energy and metabolism, signal transduction, protein synthesis and translation, matrix and structural protein, transcription control, cell cycle and DNA replication. The percentage distribution of genes in each group is 25.2, 21, 18.9, 17,

Table 1 List of signal transduction genes[1] validated the expression by real-time RT-PCR, their corresponding PCR primers[2], annealing temperature and product sizes

Group of stimulating growth factor	Gene	Primer sequence	Annealing temperature (℃)/ product size (bp)
PDGF /EGF	*abl* 1	Forward: 5′-TGAAGTTGGTGGGCTGCA-3′ Reverse: 5′-TTTTCACTGGGCCCGCA-3′	52/148
	pkC	Forward: 5′-GGCATGCCTTGTCCTGGAGA-3′ Reverse: 5′-TGTAGCCCTGCCTCGAGAGA-3′	55/149
	jak 1	Forward: 5′-CAGCCTCGCTCGTCCTT-3′ Reverse: 5′-CCAAGCCCTTCAGAGCT-3′	58/147
	raf 1	Forward: 5′-TTTCCCGGATGCCTGCTA-3′ Reverse: 5′-TCCTGCTGTCCCATGCA-3′	62/147
	h ras	Forward: 5′-TGGTGGGCAACAAGTGTGA-3′ Reverse: 5′-CCGCAATTTATGCTGCCGAA	65/150
PDGF	*pdgfrα*	Forward: 5′-TGCGGGGAAGGACTGGA-3′ Reverse: 5′-GTGAGGAGACAGCTGAGGA-3′	58/122
EGF	*eps* 8	Forward: 5′-CCGCTCCGTGGGTATGGA-3′ Reverse: 5′-ACGTCCGACACACTGCTGA-3′	54/146
TGF-β	*tgfβ* 1*i*4	Forward: 5′-GCAAGTGTGGTAGCTATCGA-3′ Reverse: 5′-CAGATTGTTCTCCTGCTCC-3′	52/136
	strap	Forward: 5′-GCCGGGAGATACAGGAGA-3′ Reverse: 5′-TGCTTATGAGCCGGGTCA-3′	52/153

[1]They are classified based on their functions as the downstream signaling molecules of the specific growth factor. [2]Primers for the internal control (β_2m) are: 5′-CATGGCTCGCTCGGTGA-3′ (forward) and 5′-AATGTGAGGCGGGTGGAA-3′ (reverse). The annealing temperature and the product size of RT-PCR for β_2m are 60℃ and 148 bp, respectively. PDGF: platelet-derived growth factor; EGF: epidermal growth factor; TGF-β: transforming growth factor-β.

12.6, 3.3, and 2, respectively. The examples of genes in each group are summarized in Table 2.

Response of signal transduction-related genes to O. viverrini ES product

A total of 59 genes/ESTs categorized by their molecular function as the signal transduction genes showed 2-fold and more up-regulation after *O. viverrini* ES product stimulation. They are the downstream signal transduction molecules for a variety of growth factors. The signal transduction genes that corresponded to the stimulation of either PDGF or EGF, PDGF, EGF and TGF-β were categorized and summarized in Table 3. The most up-regulated expressions were *tgfβ* 1*i*4 and *abl* 1 which are the representatives of TGF-β- and either PDGF- or EGF-stimulated signal transduction pathways, respectively.

In addition to the different signal transduction molecules responded to different growth factors, the receptors for PDGF/EGF and TFF-β are classified in different types as tyrosine kinase and serine-threonine kinase receptors, respectively. The cDNA array data represented the different up-regulated expression levels between these 2 types of receptors (Table 4).

Nine signal transduction genes, which responded to the specific growth factor as mentioned in Table 3, were selected and tested for their expression levels by semi-quantitative RT-PCR. The single peak of the melting curve for each gene confirmed the appropriate PCR condition (data not shown). The result indicated similar responses in both cDNA array and RT-PCR analyses of *pk*C, *jak* 1, *h ras*, *pdgfrα*, *eps* 8, *tgfβ* 1*i*4, and *strap*. However, the significant ($P < 0.05$) up-regulation was only found for those of *pk*C, *eps* 8, and *tgfβ* 1*i*4. The opposite result of mRNA expression level was detected in *abl* 1 and *raf* 1 (Figure 1).

DISCUSSION

Cell proliferation is regulated by the intricacy of cell growth regulators, signaling cascades, and mediators of cell cycle progression including cyclins, cyclin-dependent kinases (CDKs). Anti-proliferative genes coding for the apoptotic protein, cyclin/CDK inhibitors are important in controlling cell proliferation. Addition of growth factors induces cell proliferation including the fibroblasts[19]. The changes in gene expression that accompany this proliferative response have been the subject of many studies, and the responses of dozens of genes have been characterized[19]. A recent study indicated the effect of *O. viverrini* ES product in induction of fibroblast cell proliferation *in vitro*[17]. We have examined the effect of this parasitic product in the gene expression profile using a cDNA array. The NIH-3T3 cells were used because it not only had the marked response to *O. viverrini* ES product[17], but also being classified as the mesenchymal cells as the real target of *O. viverrini* ES product; the stellate cells and myofibroblasts. Among all genes/ESTs in 15K cDNA array, 885 genes showed 2-fold and more up-regulation after stimulation by *O. viverrini* ES product. Among these genes, only 536 genes had a variety of established molecular functions. Moreover, 239 of these 536 genes had cell proliferation-related functions and were primarily focused into groups based on their specific functions because of their striking increased changes (≥ 2-fold).

Most of the genes which increased their expression by *O. viverrini* ES product stimulation were the proteins controlling the enzymatic metabolism and biosynthesis. The expressions of glyceraldehyde-3-phosphate dehydrogenase, alpha-enolase and aldehyde dehydrogenase involve the glycolysis pathway. Cytochrome C oxidase and ATP synthase involve energy production from the electron transport chain. These phenomena could be easily understood because high levels of energy production and synthesis of biological macromolecules are required for the stimulation of quiescent fibroblasts by *O. viverrini* ES product into the cell cycle progression. These data suggest the possibility that ES product acts like a growth factor to stimulate cell proliferation basically by the stimulation of energy production-related gene expression[20].

In cell proliferation, the DNA replication is needed. Many proteins, including RPII215 polymerase and topoisomerase II, required for chromosome segregation at mitosis; and DNA primase, required for DNA replication, all increased their expressions in *O. viverrini* ES product-treated cells. These increased expressions will help cells undergo into cell cycle and consequently cell division. Ribonucleotide reductase is associated with the anabolic pathway of deoxynucleotide. The increased expression of this enzyme causes an increase in the deoxynucleotide triphosphate (dNTP) pool in the nucleus, which facilitates DNA synthesis and repair[21]. In addition, when cells are activated by the growth factors and then commit to enter

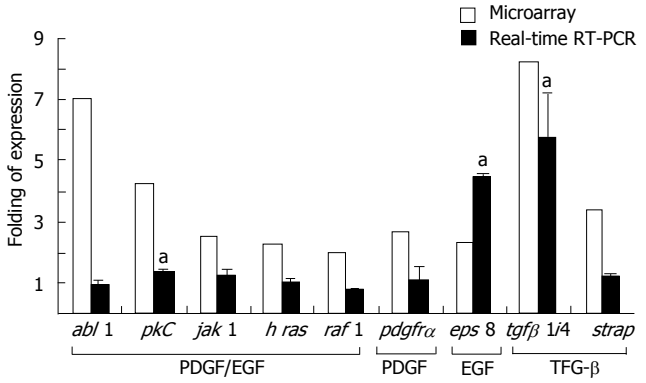


Figure 1 RT-PCR analysis of signal transduction genes up-regulation by *O. viverrini* ES product. The results of DNA array and RT-PCR are shown in comparison for each gene (mean ± SD, $^{a}P < 0.05$).

Table 2 Proliferation-related genes with 2-fold and more[1] up-regulation in fibroblast cell proliferation activated by *O. viverrini* ES product compared to the negative control (not exhaustive)

A Energy and metabolism	Glyceraldehyde-3-phosphate dehydrogenase (*gapd*), cytochrome C oxidase, alpha-enolase, ATP synthase beta subunit[2], thioredoxin, aldehyde dehydrogenase
B Signal transduction	Transforming growth factor beta-1-induced transcript 4 (*tgfβ* 1*i*4), tyrosine kinase (*abl* 1), interleukin 1 receptor-associated kinase, MAP kinase phosphatase (*mkp* 6), colony stimulating factor 3 receptor, protein kinase C, Janus kinase 2 (*Jak* 2), casein kinase I alpha (*csnk* 1*a*1), *c-myc*
C Protein synthesis and translation	Polyubiquitin C (*Ubc*) gene, ribosomal protein S27a[2], ribosomal protein L27, elongation factor Tu, RNA polymerase 1-2, translation initiation factor 4 gamma[2]
D Matrix and structural protein	Lysophospholipase 1 (*lypla* 1)
E Transcription control	Transformation/transcription domain-associated protein (*trrap*)[2], telomeric repeat binding factor 1 (*terf* 1), transcription factor EB (tcfeb), DEAD-box RNA helicase (*ddx* 21)[2]
F Cell cycle	Kinesin family member 3a (*kif* 3*a*), mitotic kinesin-linked protein 1[2], cyclin-dependent kinase 4 (*cdk* 4), *cdk* 5, cyclin B1
G DNA replication	RPII215 polymerase II large subunit, topoisomerase II alpha, DNA primase p58 subunit (*prim* 2), ribonucleotide reductase

[1]Sequence in each group is ordered from high to low expression increases; [2]represents human gene.

Table 3 Up-regulation of signal transduction-related genes in NIH-3T3 treated with *O. viverrini*, categorized in groups according to the response to the specific growth factor

Related growth factor (Accession number)	Gene name/production	Folding
PDGF/EGF		
AW544655	Tyrosine kinase (*v abl*)	7.12
C80388	Protein kinase C (*pk*C)	4.23
AU042564	Casein kinase I-alpha isoform (*csnk* 1α 1)[1]	3.81
C87299	Casein kinase I-epsilon (*csnk* 1ε)	2.88
AU046274	Ras-GTPase activating protein SH3-domain binding protein (*c3bp*-pending)	2.73
C87788	*JAK*1 protein tyrosine kinase	2.56
AU017366	*H ras*	2.26
AW552623	*N ras*	2.01
PDGF		
AW537708	PDGF receptor, alpha-polypeptide (*pdgfr* α)	2.64
EGF		
C88280	Epidermal growth factor receptor pathway substrate (*eps* 8)	2.32
TGF-β		
AW546174	Transforming growth factor beta-1-induced transcript 4 (tgfβ 1*i*4)	8.24
AU016757	*c myc*	3.70
C79202	Serine/threonine kinase receptor-associated protein (*strap*)	3.40

[1]Represents human gene.

Table 4 cDNA array analysis showing up-regulation of genes encoded for the kinase receptors

Receptor kinases	Gene names (accession number)	Folding
Serine-threonine kinase	Serine/threonine kinase 11 (C85710)	4.42
	Serine/threonine kinase 19 (AW557191)	3.28
	Serine/threonine kinase 4 (AU020804)	2.88
Tyrosine kinase	TYRO3 protein tyrosine kinase 3 (AW556118)	2.60
	JAK1 protein tyrosine kinase (C87788)	2.56
	Protein tyrosine kinase 9 (AW544421)	1.83
	Downstream of tyrosine kinase 1 (AW557123)	1.59

the cell cycle, the cascade of gene expression within the cells is needed. This means it is important that the initial gene expressed to be protein for the expression of the later ones. This is the action of protein classified as the transcription factor. In cells treated with *O. viverrini* ES products, a variety of transcription factors are activated.

Cyclins and CDKs are the important proteins in cell cycle progression[22]. The response of cell to *O. viverrini* ES product indicated the increased expression of many CDKs and cyclins. CDK4 and 5 are important at the G1 phase, whereas cyclin B1 is crucial in G2 phase. The increased expression of phosphorylated cyclin D1 was reported in fibroblast proliferation induced by *O. viverrini* ES product[17], thereby supporting the increased expression of

CDK4 in this study. This result supports the importance of cyclin D1-CDK4 expression in the re-entry of the serum-stimulated fibroblasts into the cell-division cycle[23]. Moreover, *O. viverrini* ES product induced the expression of kinesins which are a family of microtubles and are involved in many crucial cellular processes including cell division[24]. The kinesin family member 3a (*kif 3a*) and mitotic kinesin-like protein are crucial for spindle assembly and function, chromosome segregation, mitotic checkpoint control, and cytokinesis in higher eukaryotes[25]. Cells cannot divide without the appropriate functions of these genes.

Rapid and efficient production of macromolecule in protein synthesis and degradation of superfluous proteins play important role in cell cycle progression[26]. The increased RNA polymerase which determines the efficiency of mRNA production supports the capability of other genes to increase their expressions. The ribosomal proteins as the component of ribosomes, translation initiation factors and elongation factors increased their expressions which normally occur to promote proteins synthesized for cell division. In addition, the expression of polyubiquitin C (*ubc*) increased to get rid of the superfluous proteins already synthesized and that have reached their expired period. The increased expression of these genes is crucial for the turnover of the appropriate proteins for the right stimuli at the right time.

Matrix and structural proteins are important in supporting cell proliferation[27]. In this study, some matrix and structural proteins increased their expression level by *O. viverrini* ES product stimulation. Lysophospholipase D (lysoPLD) catalyses the production of lysophosphatidic acid (LPA) from the lysophosphatidylcholine (LPC). LPA has been demonstrated to be a potent inducer of cell proliferation of multiple cell lineages[28]. Taken together, all the data presented in this study confirm that *O. viverrini* ES product can activate cell proliferation resulting in the response of gene expression profile like a serum or growth factor[29].

Array analysis is not only crucial in understanding the effect of the ES product on cells but also to predict what should be the growth factor available in *O. viverrini* ES product. In this work, we took the advantage of this technology to decipher the possible genome expression circuit induced by *O. viverrini* ES product. The comparison of *O. viverrini* ES product-induced gene expression profile with that of established growth factor profiles may be useful to know the cell signaling pathway utilized by *O. viverrini* ES product in induction of cell proliferation. The *O. viverrini* ES product stimulated cell proliferation like the response of cells to the calf serum[19]. There are many types of growth factors contained in the calf serum including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-β, and insulin-liked growth factor (IGF). Each of them stimulates a different signal transduction profile which is the most important feature used to distinguish the types of growth factor-stimulated cell responses[30,31]. In this study, the signal transduction genes selected from the array data can be categorized based on their functions as the downstream signal transduction molecules of both PDGF and EGF (PDGF/EGF), only PDGF, only EGF, and only TGF-β, according to the previous reports[30,31]. Though PDGF and EGF are different growth factors using different cell surface receptors, they used, in part, the same signal transduction pathway[32] including *abl* 1, *pk*C, *csnk* 1*a*1, *ras* and *jak* 1. The results of this study showed that *O. viverrini* ES product stimulated the expression of these genes. It may be possible that *O. viverrini* ES product activated cell proliferation *via* PDGF/EGF-driven signaling pathway. Moreover, *pdgfrα*, *eps* 8, and *tgfβ* 1*i*4, the specific genes for PDGF, EGF, and TGF-β, respectively, were found to be up-regulated as well. Based on the increased expression, *tgfβ* 1*i*4 was ranked in the uppermost expression level. It is supposed that *O. viverrini* ES product stimulated cell proliferation *via* TGF-β signaling pathway as well.

The difference between signal transduction pathways activated by TGF-β and PDGF/EGF is mainly focused on their different types of receptors. The receptors of TGF-β are membrane-bound receptors exhibiting intrinsic serine-threonine kinase activities, whereas the tyrosine kinase receptor is activated by EGF or PDGF[30,31]. From cDNA array data, the activation of serine-threonine kinase and tyrosine kinase receptors encoded gene expression showed the involvement of both types of receptors in the stimulation by *O. viverrini* ES product. This evidence still supports the possibility that TGF-β and EGF/PDGF can be the candidate signal transduction pathway induced by *O. viverrini* ES product.

Since the uncertain mRNA expression level was detected by cDNA array analysis, RT-PCR was used to measure the exact expression. From the list of up-regulated signal transduction-related genes, we selected 9 genes for additional analysis to validate the array expression data. The result showed marked increase of *tgfβ* 1*i*4 expression. It has been proven that *tgfβ* 1*i*4 is a direct target of TGF-β[33,34] and also is the transcriptional modulator stimulated by TGF-β. The *tgfβ* 1*i*4 gene expression was transcriptionally activated by TGF-β, phorbol 12-myristate 13-acetate, and serum but not appreciably by EGF[34]. The up-regulation of *tgfβ 1i4* expression induced by *O. viverrini* ES products may indicate the stimulation of the TGF-β-activated signal transduction pathway. The normal expression of *strap*, the inhibitor of the TGF-β signal transduction cascade[35], supports this conclusion.

For the EGF and PDGF-stimulated signal transduction pathways, the expressions of the common genes, including *abl* 1, *jak* 1, *h ras* and *raf* 1, were not significantly increased. Moreover, the specific gene related to PDGF-stimulated signal transduction, *pdgfrα*, was expressed at the same level as in cells without *O. viverrini* ES product treatment. This result excludes the potential of *O. viverrini* ES product in stimulating cell proliferation through the PDGF-mediated signal transduction pathway. For the EGF-stimulated signal transduction pathway, *eps* 8 is the transcription factor required in the EGF-stimulated cell proliferation and is confirmed to be the gene product that represents a novel substrate for tyrosine kinase receptors[36]. Adoptive expression of *eps* 8 cDNA in fibroblastic or hematopoietic target cells expressing the EGFR resulted in an increased mitogenic response to EGF, implicating the *eps* 8 product

in control of mitogenic signals activated by EGF[36]. That the *O. viverrini* ES product markedly stimulated the expression of this gene represents the possibility that this parasitic product stimulates cell proliferation *via* EGF-mediated signal transduction cascade. There are 4 main pathways induced by EGF, including the activations of Ras/Raf/ERK, phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ (PLC-γ)/protein kinase C (PKC) and c *jun*-N-terminal kinase (JNK). These pathways lead to different cellular actions[37]. Only the Ras/Raf/ERK and JNK-transduced signal transduction pathways involve cell proliferation. In this study, the expression of *h ras* and *raf* 1 was slightly up-regulated by *O. viverrini* ES product, indicating *O. viverr*ini ES product stimulates cell proliferation *via* other pathways rather than the common pathway of Ras/Raf/ERK. The up-regulated expression of *pk*C represents the possibility of cell proliferation activated by *O. viverrini* ES product *via* the EGF-stimulated PKC signal transduction pathway. This is opposed to the data that *pk*C-mediated signal transduction pathway stimulated by EGF does not involve in control of cell proliferation[37]. Moreover, the array data showed the increased expression of JNK (2.78-fold, data not shown) represents the possibility that EGF-associated JNK pathway may be the pathway activated by *O. viverrini* ES product.

Taken all together, *O. viverrini* ES product activates cell proliferation *via* either TGF-β- or EGF-mediated signal transduction pathways. These two signal pathways have been proved to be strongly correlated with cancer development[37,38]. TGF-β also mediates tumor-promoting effects, through differential effects either on tumor or stromal cells[38]. Its signaling pathway is being evaluated as a prognostic or predictive marker for cancer patients. Over-expression of EGF has been found in many cancer types[37]. The roles of TGF-β and EGF in cholangiocarcinogenesis need further investigations. Regardless of the exact signal transduction pathway stimulated by *O. viverrini* ES product, the induction of fibroblast cell proliferation and the accumulation of extracellular matrix (i.e. collagen) have been proven to be associated with the fibrogenetic process[13]. The increased expression of collagen type I, III, and IV (between 2-3-fold increase) was observed in cDNA array analysis (data not shown). This supports the hypothesis that *O. viverrini* ES product can stimulate cell proliferation and collagen production leading to the fibrosis formation. Since the fibrosis has been detected in cholangiocarcinoma in both animal models and human[9-11], it is possible that *O. viverrini* ES product-induced fibrosis may be one component in cholangiocarcinogenesis. Moreover, it may be proposed that the ES product-induced fibrosis may act synergistically with the immunological response against the parasites to promote CC.

In summary, this study demonstrates the gene expression profile obtained from cDNA array analysis to determine the possibility of *O. viverrini* ES product as the growth factor in induction of fibroblast cell proliferation. Signal transduction-related genes may have a significant expression increment in cells exposed to this parasitic product. Among this set of genes, *tgfβ* 1*i*4 and *eps* 8 were found to have the strongest expression levels with corroboration data obtained by both cDNA array and RT-PCR. These two genes are the specific signal transduction molecules for TGF-β- and EGF-stimulated pathways. It may therefore be proposed that *O. viverrini* ES product activated cellular proliferation *via* the pathways of these growth factors. The issues of whether one type of growth factor is capable to cross-stimulate the two pathways or two types of growth factors are available in the *O. viverrini* ES product, needs future experiments to be clearly elucidated. This report is an important resource to indicate the mechanism of *O. viverrini* ES product-stimulated fibroblast cell proliferation *via* TGF-β- or EGF-stimulated signal transduction pathways. Since the activated fibroblast has been shown to initiate non-tumorigenic epithelium to carcinoma[16], *O. viverrini* ES product-stimulated fibroblast may play a part to change bile duct epithelium into CC. The ongoing research in understanding the signaling pathways stimulated by *O. viverrini* ES product and roles of fibroblasts in the development of CC will provide a novel target for chemoprevention and treatment of fibrosis in this cancer which may delay the formation of CC.

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CLINICAL RESEARCH

Effect of abdominal trauma on hemorrhagic shock-induced acute lung injury in rats

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Abstract

AIM: To evaluate the effects of abdominal trauma on hemorrhagic shock-induced acute lung injury in rats.

METHODS: Five groups were allocated (n = 8) in the study. Group I was taken as the control group, group II as the hemorrhagic shock group, group III as hemorrhagic shock + laparotomy, group IV as hemorrhagic shock + splenectomy and group V as splenectomy + omentectomy + hemorrhagic shock group. Hemorrhagic shock was induced by drawing blood and reducing mean arterial pressure (MAP) to 40 mmHg within 10 min. After a hypotensive period of 1 h, animals were resuscitated. Bronchoalveolar lavage (BAL) was performed to recover cells from the alveolar space with 40 mL of BAL fluid after resuscitation malondialdehyde (MDA) and L-γ-glutamyl-L-cysteinyl-glycine (GSH) levels were measured in serum, erythrocytes and lung tissue.

RESULTS: Serum, erythrocyte, lung tissue MDA and GSH levels were significantly increased in hemorrhagic shock groups II-V (P < 0.05). Lymphocyte, neutrophil and alveolar macrophage counts in BAL fluid indicated a significant difference between control and shock groups (P < 0.05).

CONCLUSION: The degree of trauma increases hemorrhagic shock-induced acute lung injury.

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Key words: Hemorrhagic shock; Acute lung injury; Abdominal trauma

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) frequently occur after major trauma with a high mortality[1,2]. Clinically, acute lung injury is characterized by altered gas exchange, dyspnea, decreased static compliance, and nonhydrostatic pulmonary edema[3]. Firmly implicated mediators are endotoxin, cytokines, and products of arachidonic acid metabolism, leukocyte-derived proteolytic enzymes, and toxic oxygen species. The basic mechanisms of lung injury are focused on oxidant-mediated damage[4]. Recent evidence supports that leukocyte activation and the subsequent release of large quantities of highly reactive oxygen radicals and hydrogen peroxide are partially responsible for diffuse microvascular and tissue injury in patients with hemorrhagic shock. It has been shown that neutrophils in trauma patients release greater amounts of superoxide anion, proteases, and proinflamatory cytokine[5]. In addition to neutrophils, alveolar macrophages and circulating macrophages as well as endothelial cells aggravate lung injury and alveolar neutrophil sequestration[6]. In response to various inflammatory stimuli, lung endothelial cells, alveolar cells, airway epithelial cells, and alveolar macrophages, produce both nitric oxide and superoxide[7].

To address these issues we designed a trauma model of controlled hemorrhagic shock. Free oxygen radical metabolism of serum, erythrocyte, lung tissue and cellular change in alveolar epithelium was investigated in this model, but the main objective was to understand the role of abdominal injury in hemorrhagic shock.

MATERIALS AND METHODS

Animals

Adult male Wistar Albino rats weighing 243-325 g were used in experiments. Animals were housed and kept at 25℃ in a 12 h light/dark cycle and used in studies after an acclimatization period of 7 d. Rats were allowed to have

Table 1 Serum, erythrocyte, tissue MDA and GSH levels, and lymphocyte, neutrophil, alveolar macrophage counts in BAL fluid of different groups (mean ± SD)

	Group I	Group II	Group III	Group IV	Group V
Serum MDA (μmol/L)	1.374 ± 0.24[a]	2.095 ± 0.246	2.54 ± 0.18	3.04 ± 0.12	3.57 ± 0.23[c]
Serum GSH (μmol/L)	29.005 ± 4.14	15.374 ± 4.369	11.149 ± 1.71	8.240 ± 2.65	5.055 ± 0.86
Erythrocyte MDA (μmol/L)	411.048 ± 31.61[a]	665.676 ± 173.609	723.287 ± 89.0	792.28 ± 235.6	900.28 ± 254.9[c]
Erythrocyte GSH (μmol/L)	74.145 ± 7.35	52.702 ± 10.725	52.412 ± 9.684	46.831 ± 6.408	27.852 ± 10.438
Tissue MDA (μmol/L)	11.721 ± 1.79[a]	16.651 ± 3.702	17.315 ± 1.485	18.178 ± 2.364	20.327 ± 5.786[c]
Tissue GSH (μmol/L)	0.318 ± 0.053	0.170 ± 0.056	0.101 ± 0.020	0.082 ± 0.001	0.019 ± 0.001
Lymphocyte/100 cells	0.0 ± 0.0	0.5 ± 1.07	1.0 ± 0.0	2.75 ± 0.46	3.5 ± 1.07
Neutrophil/100 cells	2.88 ± 0.35	1.88 ± 1.36	2.63 ± 0.74	3.38 ± 0.74	4.75 ± 0.89
Alveolar macrophage/100 cells	1.38 ± 0.52	4.0 ± 2.27	4.57 ± 1.58	3.25 ± 0.71	3.63 ± 0.52

[a]$P < 0.05$ *vs* hemorrhagic shock groups; [c]$P < 0.05$ *vs* other groups.

free access to rodent chow and water before experiment. All animals were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. The Süleyman Demirel University Medical School Animal Use and Care Committee approved all experiments. In the experiments, forty rats were used and allocated into five groups ($n = 8$). Group I was taken as the control group, group II as the hemorrhagic shock group, group III as hemorrhagic shock + laparotomy group, group IV as splenectomy + hemorrhagic group, and group V as splenectomy + omentectomy + hemorrhagic shock group.

Methods

Animals were intraperitoneally anesthetized with 80 mg/kg ketamine and 8 mg/kg xylazine. The right carotid artery was cannulated with a 24-gauge angiocathether for monitoring the mean arterial pressure (MAP), blood sampling, and resuscitation. Hemorrhagic shock was induced by drawing blood and reducing MAP to 40 mmHg for 10 min. Blood was collected into 0.1 mL citrate to prevent clotting. After a hypotensive period of 1 h, animals were resuscitated by transfusion of blood and Ringer lactate in a volume equal to that of blood. After a period of two hours blood samples were taken *via* carotid artery, and the catheter was removed with the artery ligated. The cervical incision was extended towards midline to isolate trachea. Trachea was catheterized with a feeding tube (No: 8) and stabilized with 4/0 silk suture to perform bronchoalveolar lavage (BAL). BAL was performed to recover cells from the alveolar space with 40 mL of BAL fluid. Lung tissue was resected to measure tissue malondialdehyde (MDA) and L-γ-glutamyl-L-cysteinyl-glycine (GSH) levels after bronchoalveolar lavage. Control animals underwent the same procedures, but hemorrhage was not induced.

In groups III-V, additional surgical procedures were added. In group III (hemorrhagic shock + laparotomy) after hemorrhagic shock was maintained, a midline incision in 2 cm length was performed on the abdominal wall. The incision was sutured with 4/0 silk suture. In group IV (hemorrhagic shock + laparotomy + splenectomy) after laparotomy splenectomy was performed with its vessels ligated, laparotomy incision was sutured as in group III. In group V (hemorrhagic shock + laparotomy + splenectomy + omentectomy) omentectomy was added. All vessel boundaries were ligated when the omentum was removed.

BAL fluid and its analysis

The lungs were lavaged using the feeding tube with BAL fluid containing cold phosphate buffered saline (PBS), 8 mmol/L sodium phosphate, 2 mmol/L potassium phosphate, 0.14 mol/L sodium chloride, and 0.01 mol/L potassium chloride (pH7.4) and 0.1 mmol/L ethylenedia minetetraacetic acid (EDTA). Forty mL of BAL solution was used for lavage in each animal, and about 90% was re-collected. The fluid was centrifuged at 700 r/min for 15 min. Supernatant was collected and re-suspended in 1 mL of the same solution. Two slides were prepared from each sample, and stained with Wright-Giemsa stain. A total number of 100 cells were counted in each slide with immersion objective (100 ×) and the number of neutrophils, lymphocytes and alveolar macrophages was counted respectively.

MDA and GSH levels in serum, erythrocyte and lung tissue

Hemolysate and serum of the blood specimens were prepared and stored at -70℃ until assay. The lung specimens were homogenized with a mechanic homogenizator (Ultra-Turrax T25) and sonicated (Bandelin D.12207) in phosphate buffered saline (pH7.4). The homogenate was centrifuged at 10000 r/min for 15 min at 4℃ and supernatant was used for determination of the MDA and GSH concentrations. MDA, an end product of lipid peroxidation, was assayed by the method of Drapper and Hadley[8]. GSH levels were determinated by the method of Beutler *et al*[9].

Statistical analysis

All data were analyzed by one-way ANOVA with *post hoc* test Bonferroni. Data were expressed as mean ± SD. $P \leqslant 0.05$ was considered statistically significant.

RESULTS

There was no difference in MAP values before and after hemorrhagic shock between the groups ($P > 0.05$). Serum, erythrocyte and lung tissue MDA levels were significantly higher in hemorrhagic shock groups (groups II-V) than in control group ($P < 0.05$, Table 1). MDA levels were also significantly different in groups II-V. Serum MDA

levels had a trend to increase with hemorrhagic shock and additional surgical injury ($P < 0.05$). The same trend could be seen in erythrocyte and lung tissue MDA levels ($P < 0.05$, Table 1).

Serum, erythrocyte and lung tissue GSH values were significantly lower in groups II-V than in control group ($P < 0.05$, Table 1). All GSH levels were decreased especially in group V ($P < 0.05$), but the difference in groups II-IV was not significant ($P > 0.05$). The decreased serum GSH levels were parallel to the severity of trauma, but the difference in groups II-V was not statistically significant ($P > 0.05$, Table 1). Similar GSH levels in lung tissue were obtained. Tissue GSH level in group V was significantly lower than that in other groups ($P < 0.05$).

Lymphocyte, neutrophil and alveolar macrophage counts in BAL fluid indicated a significant difference between groups ($P < 0.05$, Table 1). The highest lymphocyte and neutrophil counts were found in group V. Although there was no significant difference in group II-V, lymphocyte, neutrophil and alveolar macrophage counts were significantly higher in groups II-V than in control group.

DISCUSSION

Many mediators of acute lung injury in animal models and ARDS in human beings have been implicated as causative factors. Selection of agents that can abrogate these mediators is based on the efficacy of these agents in whole animal models, and their relative potency and potential toxicity. Through examination of metabolic processes, GSH has been shown to be important in host defenses against oxidative stress[3]. Another important agent showing oxidative stress is MDA, a marker of free radical activity[3]. After ischemia-reperfusion, lipid peroxides induce a substantial drop in intracellular GSH levels[10,11]. In this study, hemorrhagic shock and resuscitation significantly elevated serum, erythrocyte and lung tissue MDA levels ($P < 0.05$). GSH levels were significantly lower in hemorrhagic shock groups than in control group ($P < 0.05$). It was reported that oxidative stress significantly elevated MDA levels and reduced GSH levels[12,13]. These significant alterations were also seen in our study. MDA levels were significantly higher in group V (laparotomy + splenectomy + omentectomy) than in other groups ($P < 0.05$). Serum GSH levels of erythrocyte and lung tissue were also altered in hemorrhagic shock groups ($P < 0.05$). All these findings support the hypothesis that ischemia-reperfusion affects free oxygen radical metabolism and additional surgical trauma increases this effect. Lipid peroxidation seems the main mechanism of free radical toxicity and results in alterations of structural integrity and function of cellular membranes[14]. Intracellular overproduction is not the only mechanism of free radical-induced lung injury[15]. Hypoxia can damage alveolar macrophages, which may release chemotactic and activating factors for polymorphonuclear neutrophils[16]. The release of toxic oxygen may result in endothelial injury, which is an early event in the course of oxygen-induced lung damage.

Neutrophils sequestered in the lung play a central role in the pathogenesis of ARDS. Lung polymorphonuclear neutrophile accumulation in ARDS occurs as a result of a cascade of cellular events initiated by either infectious or noninfectious inflammatory stimuli[17]. Neutrophil and lymphocyte counts were significantly increased in BAL fluid of hemorrhagic shock groups in this study ($P < 0.05$). There was also a significant difference between these groups. The count of these cells increased significantly with the degree of surgical trauma ($P < 0.05$). Alveolar macrophage count was similarly increased in BAL fluid in shock groups ($P < 0.05$), the difference between these groups was not significant. There was no relation between the degree of trauma and alveolar macrophage count increase ($P > 0.05$). These observations can be explained with the findings in the studies of Jorens *et al*[18] and Bernard *et al*[19]. Acute lung injury in ARDS is largely due to activated neutrophils, which not only aggravate oxidative stress in the lungs but also release mediators, including cytokines and lipids.

In conclusion, oxidative stress and surgery increase free oxygen radicals in serum, erythrocytes and lung tissue and lead to epithelial injury in the lungs. The observed injury seems related with the degree of trauma.

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RAPID COMMUNICATION

The vital threat of an upper gastrointestinal bleeding: Risk factor analysis of 121 consecutive patients

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Abstract

AIM: To analyze the importance in predicting patients risk of mortality due to upper gastrointestinal (UGI) bleeding under today's therapeutic regimen.

METHODS: From 1998 to 2001, 121 patients with the diagnosis of UGI bleeding were treated in our hospital. Based on the patients' data, a retrospective multivariate data analysis with initially more than 270 single factors was performed. Subsequently, the following potential risk factors underwent a logistic regression analysis: age, gender, initial hemoglobin, coumarines, liver cirrhosis, prothrombin time (PT), gastric ulcer (small curvature), duodenal ulcer (bulbus back wall), Forrest classification, vascular stump, variceal bleeding, Mallory-Weiss syndrome, RBC substitution, recurrent bleeding, conservative and surgical therapy.

RESULTS: Seventy male (58%) and 51 female (42%) patients with a median age of 70 (range: 21-96) years were treated. Their in-hospital mortality was 14%. While 12% (11/91) of the patients died after conservative therapy, 20% (6/30) died after undergoing surgical therapy. UGI bleeding occurred due to duodenal ulcer (n = 36; 30%), gastric ulcer (n = 35; 29%), esophageal varicosis (n = 12; 10%), Mallory-Weiss syndrome (n = 8; 7%), erosive lesions of the mucosa (n = 20; 17%), cancer (n = 5; 4%), coagulopathy (n = 4; 3%), lymphoma (n = 2; 2%), benign tumor (n = 2; 2%) and unknown reason (n = 1; 1%). A logistic regression analysis of all aforementioned factors revealed that liver cirrhosis and duodenal ulcer (bulbus back wall) were associated risk factors for a fatal course after UGI bleeding. Prior to endoscopy, only liver cirrhosis was an assessable risk factor. Thereafter, liver cirrhosis, the location of a bleeding ulcer (bulbus back wall) and patients' gender (male) were of prognostic importance for the clinical outcome (mortality) of patients with a bleeding ulcer.

CONCLUSION: Most prognostic parameters used in clinical routine today are not reliable enough in predicting a patient's vital threat posed by an UGI bleeding. Liver cirrhosis, on the other hand, is significantly more frequently associated with an increased risk to die after bleeding of an ulcer located at the posterior duodenal wall.

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Key words: UGI bleeding; Mortality; Risk factors

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INTRODUCTION

Acute bleeding from the upper gastrointestinal (UGI) tract remains a major cause of morbidity and mortality. Over the last decades, mortality after UGI bleeding has been reported to range between 8% and 14% of cases[1-8]. Selected groups, i.e. patients who underwent surgical therapy for UGI bleeding, die at a risk of about 21%[9].

Despite improved diagnostic measures, emergency endoscopy[10], improved conservative and surgical treatment[5,11], mortality after UGI bleeding remains high[1]. An increase in the number of high-risk patients with reduced ability to compensate for the consequences of bleeding may be one of the reasons for this phenomenon[11,12]. Further, UGI bleeding is the most common emergency in gastroenterology, with an incidence of about 50 to 150 bleeding episodes per 100 000 inhabitants per year[4,11,13,14].

Numerous prognostic factors have been described in literature to be associated with a lethal outcome; however, to date, it remains unclear whether a single or a combination of these parameters is valid in predicting the vital threat of a patient with an UGI bleeding[1,13,15,16]. Some

factors were assessed for decision making on early surgical therapy in patients with bleeding ulcers[5,17]. However, they have not become clinical routine due to their poor reliability.

There are very few studies which report on risk factors for an increased threat after UGI bleeding based on multivariate analysis[1]. Thus, our retrospective study was designed to evaluate published risk factors for their value in predicting the threat to patients with UGI bleeding.

MATERIALS AND METHODS

Patients

A database was established retrospectively using information on consecutive admitted patients, with evidence of UGI bleeding treated in the Department of General Surgery, Ruprecht-Karls-University, Heidelberg, Germany during 4 consecutive years from January 1st, 1998 to December 31st, 2001.

The patients were identified with endoscopy, patients' admission (electronic patients chart SARA-Med), and an operation protocol database. Within the evaluated 4 years, a total of 264 patients with the suspicion of UGI bleeding were found (Figure 1). Thirty-six of these were treated temporarily in the outpatient clinic. In these cases no hospitalization was necessary. Further 38 patients were excluded from analysis since their charts could not show enough evidence of an UGI bleeding. Forty-two patients' charts were not available for analysis. Thus, these patients were excluded from further analysis. Twenty-six cases have not been documented in the way needed for an appropriate analysis. Since a gap-free documentation is needed for statistics, a total of 65 patients were excluded from this study. Moreover, patients were excluded from this study if UGI bleeding was due to previous surgery or endoscopic intervention (n = 1).

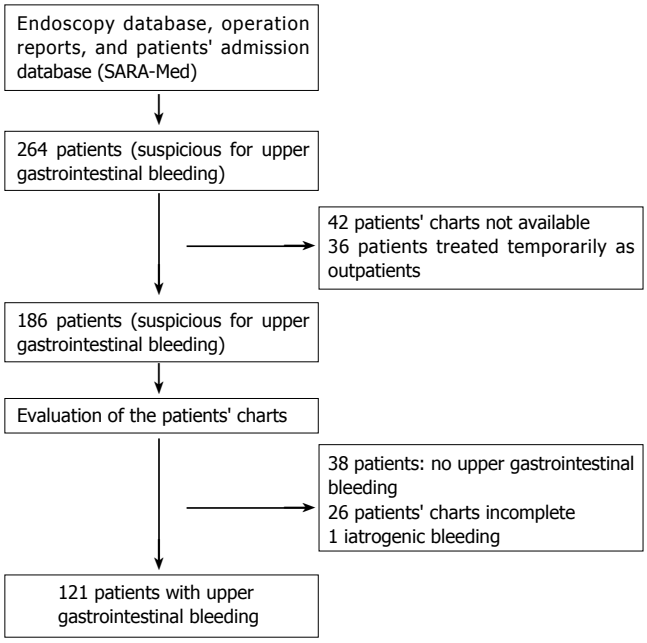


Figure 1 Identification of patients with an UGI bleeding qualifying for statistical analysis (Identification of all patients with an UGI bleeding who received clinical treatment within a period of 4 years from January 1st, 1998 to December 31st, 2001).

Literature research

MedLine was searched for gastrointestinal hemorrhage, gastrointestinal bleeding, bleeding peptic ulcer, peptic ulcer hemorrhage, bleeding from the upper gastrointestinal tract, stigmata of hemorrhage, prediction of mortality, emergency endoscopy, predictive factors, and further hemorrhage. Fifty-five publications on prognostic factors and risk factors of UGI bleeding were found, including papers mentioned in their references. Subsequently, factors (i.e., renal failure, heart failure, diabetes mellitus and others; n = 79) were classified according to the frequency of their appearance. Factors which fulfill at least one of the following conditions have been included: Factors which have been mentioned at least twice in the context of risks and prognosis for mortality, recurrence of bleeding, failed endoscopic therapy or necessity of surgery and a significant correlation between the parameter and mortality after univariate or multivariate analysis.

Analysis at different time points of therapy

During various stages of therapy, different information was available. Thus, analysis was performed at the time of admission, right after endoscopy and after completed therapy. The following information on the patients were given and analyzed:

At admission: Age, gender, medication [i.e. coumarines, acetyl salicylic acid (ASS), non-steroidal antirheumatics (NSA)], liver disease, laboratory findings (i.e. hemoglobin, PT) were recorded.

At endoscopy: In addition to information obtained at admission, the localization, the Forrest classification and the presence of a vascular stump were elucidated.

At the end of therapy: In addition to the aforementioned information, the number of blood units transfused, frequency of re-bleeding and the number of operations were recorded.

Statistical analysis

The in-hospital survival was chosen as the endpoint. It was analyzed as to how the probability of survival depends on covariates by means of stepwise logistic regression[18] where the significance level for entry of a covariate was chosen as 0.2 and the significance level for staying was chosen as 0.15.

Only parameters which were documented for all of our 121 patients with a special focus on clinical relevant parameters published before were analyzed. The possible covariates which were considered are: gender, age (dichotomised at 70 years), medication (ASS, NSA, and coumarines), liver cirrhosis, gastric ulcer (small curvature), duodenal ulcer (bulbus back wall), varices, Forrest classification, vascular stump, prothrombin time (dichotomised at 50%), hemoglobin concentration (dichotomised at 60 g/L), transfused blood units (dichotomised at 6 units), recurrence of bleeding and operations.

Three models were applied. The first considered the knowledge of covariates before the first gastroduodenoscopy. The second model takes only the patients with

Table 1 Reason for the occurrence of upper gastrointestinal (UGI) bleeding

Reason for bleeding	Duodenal ulcer	Gastric ulcer	Erosive mucosal lesion	Esophageal varicosis	Mallory-Weiss-syndrome	Cancer	Coagulopathy	Lymphoma	Benign tumor	Unknown reason
%	29.8	28.9	16.5	9.9	6.6	4.1	3.3	1.7	1.7	0.8
Number (n = 125)[1]	36	35	20	12	8	5	4	2	2	1

[1]Four patients presented with both duodenal and gastric ulcers with each showing an endoscopic evidence of bleeding and thus each ulcer was counted as a whole.

Table 2 Logistic regressions analysis of covariables recorded at admission of 121 patients considering their ability in predicting the patients' lethality

Covariable	*P*	Statistical inclusion in model	Odds ratio	95% confidence interval
Cirrhotic liver disease	0.005	Yes	4.5	1.4-13.8
Hb	0.256	No		
Medication	0.419	No		
PT	0.672	No		
Age	0.822	No		
Gender	0.879	No		

bleeding in the ulcers where the Forrest classification is valid into account, whereas the third model considers the covariates which are known after the termination of bleeding[18].

RESULTS

Patients

The total of 121 patients with UGI bleeding included in our analysis comprised of 70 males (58%) and 51 females (42%), making a gender distribution of 1.4:1. The median age distribution of the patients was 70 years, the youngest being 21 and the oldest 96 years. The most frequent cause for UGI bleeding was duodenal ulcer (n = 36; 30%) of which n = 13 were located at the bulbus back wall. Other reasons for UGI bleeding in descending order of occurrence were gastric ulcer (n = 35; 29%) of which n = 12 were located at the lesser curvature, erosive lesions of the mucosa (n = 20; 17%), esophageal varicosis (n = 12; 10%), Mallory-Weiss syndrome (n = 8; 7%), cancer (n = 5; 4%), coagulopathy (n = 4; 3%), lymphoma (n = 2; 2%), benign tumor (n = 2; 2%) and an unknown reason (n = 1; 1%). Four patients had both duodenal and gastric ulcers with each ulcer showing endoscopic evidence of bleeding, and hence each ulcer was counted as one (Table 1).

Total lethality

Out of the 121 patients analyzed, 17 died, resulting in a total mortality of 14%.

Logistic regression analyzed at different time points of therapy

At admission: Of all the prognostic parameters which were considered for logistic regression, only the prognostic parameter of cirrhotic liver disease could improve the predictability of dying as a result of an UGI bleeding. Liver cirrhosis at admission increased the risk of mortality due to UGI bleeding by 4.5 fold (P = 0.005, 95%-confidence interval: 1.4-13.8). The other prognostic parameters recorded at admission (Hb, PT, age, gender and medication) did not have a predictive value (Table 2).

At endoscopy: Sixty-seven patients with duodenal or gastric ulcers were analyzed; fourteen prognostic parameters which were recorded during endosopic examination were tested on how far they prove to be a threat to the patients' survival. Three of the tested parameters were taken up into the model, as their existence could help in predicting the faith of an ulcer patient. Cirrhotic liver disease (P = 0.029; 95%-confidence interval: 1.1-34.4) increased the mortality risk 5.9-fold as a result of UGI bleeding. Furthermore, male patients had a 4.8-fold higher risk of not surviving an UGI bleeding (P = 0.065; 95%-confidence interval: 1.0-34.8). An ulcer located at the bulbus back wall increased a patient's lethality 3.5-fold (P = 0.135; 95%-confidence interval: 0.6-20.7). Other parameters, such as the Forrest classification, played no substantial role in improving the predictive strength of the statistic model (Table 3).

At the end of therapy: Only 2 of the 12 different prognostic parameters tested at the end of therapy were of significance; a patient with cirrhotic liver disease had a 5.7-fold higher risk of dying of an UGI bleeding (P = 0.005; 95% confidence interval: 1.7-19.3) as compared with a patient with healthy liver. An ulcer located at the bulbus back wall increased the lethality 3.4-fold (P = 0.092; 95%-confidence interval: 0.7-14.7). All remaining parameters did not improve the predictability and were not taken up in model (Table 4).

Subgroup analysis of liver disease patients

The logistic regression analysis at the 3 different time points showed that the covariable cirrhotic liver disease significantly increased the predictive value at all time points. In 21 of our evaluated patients liver disease occurred, analysis of the correlation between these patients and 9 covariables showed following findings: an initial Hb < 6 mg/dL increased the risk of dying of an UGI bleeding 9.75-folds (P = 0.077; 95%-confidence interval: 0.8-121.8). When these patients experienced recurrence bleeding during their hospital stay, the risk was increased 4.5-folds (P = 0.135, 95% confidence interval: 0.6-32.3). Moreover, additional surgical treatment resulted in a 4.5-fold risk increase (P = 0.164; 95% confidence interval: 0.5-37.4). On the contrary, the necessity to transfuse more that 6 units red blood cells (RBCs) as well as the location of a gastric ulcer at the small curvature did not increase the risk factor (Table 5).

Table 3 A logistic regression analysis evaluating 14 prognostic parameters in their ability to increase the predictability of lethality of 67 patients with UGI bleeding resulting from duodenal or gastric ulcer

Covariable	*P*	Statistical inclusion in model	Odds ratio	95% confidence interval
Cirrhotic liver disease	0.029	Yes	5.9	1.1-34.4
Gender	0.065	Yes	4.8	1.0-34.8
Duodenal ulcer (bulbus back wall)	0.135	Yes	3.5	0.6-20.7
Forrest 1 b	0.175	No		
Forrest 2 b	0.272	No		
Hb	0.285	No		
PT	0.641	No		
Medication	0.693	No		
Forrest 3	0.721	No		
Forrest 1 a	0.748	No		
Forrest 2 a	0.782	No		
Vascular stump	0.852	No		
Age	0.926	No		
Gastric ulcer (small curvature)	0.964	No		

Table 4 Logistic regressions analysis indicating the predictive value of 12 covariables in predicting the lethality of an UGI bleeding

Covariable	*P*	Statistical inclusion in model	Odds ratio	95 % confidence interval
Cirrhotic liver disease	0.005	Yes	5.7	1.8-19.3
Duodenal ulcer (bulbus back wall)	0.092	Yes	3.4	0.7-14.7
Bleeding recurrance	0.271	No		
Hb	0.341	No		
Medication	0.345	No		
Esophageal varicosis	0.401	No		
Number of operations	0.42	No		
Substitution of RBCs	0.436	No		
PT	0.625	No		
Gastric ulcer (small curvature)	0.799	No		
Gender	0.809	No		
Age	0.863	No		

Table 5 Subgroup analysis of 21 patients with UGI bleeding and liver disease (Evaluation of the influence of 9 covariables on the lethality within the group)

Covariable	Odds ratio	95% confidence interval	*P*
Hb	9.75	0.8-121.8	0.077
Bleeding recurrence	4.5	0.6-32.3	0.135
Number of operations	4.5	0.5-37.4	0.164
Substitution of RBCs	1	0.1-7.5	1
Gastric ulcer (small curvature)	1	0.1-13.4	1
PT	0.72	0.1-5.2	0.744
Gender	0.68	0.1-5.5	0.718
Age	0.3	0.03-3.3	0.322
Esophageal varicosis	0.17	0.02-1.8	0.137

DISCUSSION

The lethality of our evaluated patient group of 14% confirms with records of selected patient groups in other surgical departments[9]. These findings compared to the standards of today's medicine where a lethality of more than 10% is classified as high, depict that the lethality of UGI bleeding patients has been underestimated tremendously. Although many risk factors are listed in literature and many scores[5,17] as well as indices[13,16] have been suggested, there is still ongoing uncertainty as to which parameters are threatening or which patient group is to be classified as high-risk group. The many univariate analysed records found in today's literature do not fully make up for the multi-factorial situation of an UGI bleeding, which requires multivariate analysis. The disadvantages of prospective study designs, when it comes to long-term studies, made us choose the limited but short-termed retrospective study design.

The results of our multivariate analysis which showed that of all the possible risk factors considered at admission of a patient with UGI bleeding, only the covariable liver cirrhosis proved to be a strong enough prognostic factor in predicting the lethality of such patients. The only multivariate study, which also evaluated patients at admission[19], reported further risk factors such as age above 75 years, diagnosed carcinoma, evidence of blood in gastric aspirate and a systolic blood pressure below 90 mmHg. These findings could not be confirmed in our study. The covariable blood in gastric aspirate was considered by us as a diagnostic criterion and hence not taken into consideration.

The analysis of data gained after endoscopy showed in our study that only the evidence of cirrhotic liver disease as covariable could significantly increase the ability to predict the lethality of a patient being treated because of UGI bleeding. Other variables, such as male gender and an ulcer located at the bulbus back wall, were found to be of weaker predictable strength. On the other hand, other studies reported an evidence of disease[20,21], recurrent bleeding[2,21,22], transfusion of more than 5 units of RBCs[20,22] and an ulcer size of more than 1 cm[20,22] as significant prognostic parameters. However, the parameters including patients age above 60 years[2,22], evidence of gastric bleeding[2], an initial Hb below 100 g/L and complications[21] as prognostic parameters applicable for a patient with ulcer bleeding could not be confirmed in our multivariate analysis.

Other multivariate analyses of prognostic parameters for patients after UGI bleeding therapy found in literature confirm our result that the covariable cirrhotic liver disease is significant in predicting the lethality of patients[13,23]. Other reports which noted factors such as elevated serum transaminases[19,23], high albumin levels[23] and existence of life-threatening disease[24] tend to confirm our findings as well, as these listed factors could be interpreted as descriptive of liver disease.

The existing correlation between liver disease and the covariables; Hb, the number of operations and recurrent

bleedings during treatment of UGI bleeding suggests their probable influence on the lethality of this patient group. The relationship could be due to the fact that these parameters tend to outline the risk profile of liver disease patients. A study which reports that the lethality of patients with liver disease correlates with the number of bleeding occurrences and the extent of renal failure[25], confirms our results.

In summary, most prognostic parameters used in clinical routine today are not reliable enough in predicting the patient's vital threat due to UGI bleeding. Liver cirrhosis is the only risk factor which shows a significantly more frequent association with a fatal course after UGI bleeding in our patients.

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RAPID COMMUNICATION

Roles of syndecan-1, bcl6 and p53 in diagnosis and prognostication of immunoproliferative small intestinal disease

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Abstract

AIM: To evaluate roles of syndecan-1, bcl6 and p53 in diagnosis and prognostication of immunoproliferative small intestinal disease (IPSID) and to study profiles of kappa (κ) and lambda (λ) light chains and IgA heavy chain.

METHODS: The study consisted of 11 cases of IPSID and similar number of controls which included 11 of normal intestinal mucosa and 11 of high grade B cell lymphoma of ileum. The parameters analyzed included clinical profiles, biochemical and other laboratory investigations, radiologic and histological findings including immunohistochemistry.

RESULTS: All IPSID cases had demonstrable serum IgA heavy chain and heavy mucosal plasma cell infiltration. According to Galian's histological staging, there were 4 patients with stage A and 7 with stage B. κ and λ light chains were over-expressed in 7 patients; 1 stage A patient had *H pylori*-positive active gastritis and eradication of *H pylori* led to disease remission. Stage A biopsies had higher expression for syndecan-1, while stage B had higher expression for bcl6 and p53. Syndecan-1, κ and λ light chains and IgA heavy chain showed inverse relationship with bcl6 and p53. All patients were treated with doxycycline. CHOP regime was added in 5 patients who developed frank lymphoma. Three died of the disease due to extensive organ infiltration.

CONCLUSION: Certain immunomarkers like syndecan-1, κ and λ light chains and IgA heavy chain could be of much help in identifying early stage IPSID. Stage B IPSID showed higher expression for bcl6 and p53 than stage A IPSID. bcl6 and p53 expressions correlated with a more advanced disease stage and aggressive tumour behavior.

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Key words: IPSID; Syndecan-1; bcl6; p53 protein; κ and λ light chains; Alpha heavy chain; *H pylori*

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INTRODUCTION

Immunoproliferative small intestinal disease (IPSID) or alpha heavy chain disease is quite frequently seen in Mediterranean countries[1-7] compared to rest of the world[8-12]. It usually affects young individual of low socio-economic status. Reduction in incidence has been observed with improvement of living conditions[13-15]. Plasma cells of IPSID secrete bio-chemically abnormal IgA heavy chain in all body fluids[16-18]. Monoclonality in plasma cell of early stage IPSID has been demonstrated by immunohistochemistry and polymerase chain reaction[19-23]. IPSID is known to have an indolent course. Complete remission could be obtained with long-term antibiotic therapy in stage A IPSID, though the ultimate outcome of the disease is unpredictable[22,23]. Syndecan-1 is a member of cell surface trans-membrane heparan sulphate proteoglycans[24] and is expressed by pre-B, mature plasma cell and myeloma cell line[25-27]. In *in vitro* studies, syndecan-1 has inhibitory effect on growth of myeloma cell line and B cell lymphoma[27,28]. Reduction in syndecan-1 expression has been observed with dedifferentiation of non-plasma cell malignant tumors[29] and down-regulation of p53 and Ki67 expression[30]. *bcl6* gene product, a nuclear phospho-protein, is expressed independently of *bcl6* gene rearrangement[31]. bcl6 expression is restricted to germinal center B cell and certain percentage of intra-follicular T cell[32]. It is over-expressed in high grade B-cell lymphoma, marginal zone B cell lymphoma and in some cases of follicular lymphoma and nodular lymphocytic predominance Hodgkin's lymphoma[31-35]. In good number of gastric MALTomas and intestinal diffuse

large B-cell lymphoma, expression patterns of bcl6 have been evaluated[36]. *p53* gene plays an important role in cell cycle by checking and controlling apoptotic pathways and abnormal cell proliferation. In absence of a functional *p53* gene, apoptotic pathway does not become activated and cell cycle progresses without the repair of the defective DNA. Hence, it is known as "gate-keeper gene"[37]. Mutation in *p53* gene results in proliferation of abnormal cell by blocking the apoptotic pathways and is seen in various pre-malignant and malignant conditions[37-39]. *H pylori*-associated early stage MALToma are known to regress following eradication of *H pylori*. This observation suggests evolvement of MALToma from benign antigen-driven B cell[40-42]. IPSID, like in *H pylori*-associated MALTomas, likely represents follicle center and/or marginal zone B-cell response to chronic infection by bacteria or parasites and eventually acquiring uncontrolled proliferation and probably monoclonality. Early staged IPSID usually responds to antibiotic therapy alone[43,44].

Histologically, Galian *et al*[23] has classified IPSID into 3 stages. Stage A is characterized by mucosal infiltrate by mature plasma cell and lymphocytes. In stage B, mucosa shows presence of atypical or immature plasma cells and lymphocytes, which could extend to deeper portion of the intestinal wall. Stage C is the frank lymphomatous stage with involvement of draining lymph nodes. Stage A is treated by antibiotics alone and stage B requires additional cytotoxic drugs[45,46]. Differentiating between stage A and B is of therapeutic and prognostic importance. With the advancement in imaging techniques, clinical staging is based on observations made on CT and MRI, without subjecting the patient to laparatomy[7]. The protocol was hence planned to study (1) histological profiles of IPSID in endoscopic biopsies, (2) to evaluate roles of syndecan-1, bcl6 and p53 in diagnosis and prognostication of IPSID, and (3) expressions of kappa (κ) and lambda (λ) light chains, and IgA heavy chain.

MATERIALS AND METHODS

Materials

The study included 11 cases of IPSID who presented with features of malabsorption. All had demonstrable serum alpha heavy chain by immuno-electrophoresis. Endoscopic biopsies of 11 patients showed heavy mucosal infiltration predominantly by plasma cell with lymphocyte. According to Galian's *et al*[23], histological grading was done as follows: stage A identified by heavy lamina propria infiltration by mature plasma cell and lymphocyte; stage B by lamina propria infiltration with extension into sub-mucosa and/or with few scatter immature plasma cell; and stage C by infiltrating immature plasma cell with variable degree of intestinal wall infiltration and regional lymph node involvement. In all patients studied, 3 to 5 mucosal biopsy fragments were available from involved segments of the intestine. Additional two tissue fragments from pyloric antrum, one each from anterior and posterior wall, were taken only when there was abnormality i.e. either involvement by the ongoing disease or if there was changes of gastritis.

Methods

Baseline assessment of all the biopsies was done on H&E-stained sections for all the eleven patients. Peroxidase anti-peroxidase technique was used for immunohistochemistry. Primary antibodies used were: (1) Syndecan-1 (dilution 1: 50, Dako), (2) bcl6 (pre-diluted, Neomarker), (3) p53 (pre-diluted, DO7, Dako), (4) κ and λ light chains (dilution 1:100, Dako), and (5) IgA specific for heavy chain (dilution 1:800, Dako). Antigen binding epitope site exposure was done by heating in a microwave oven placing the slides in a Coplin jar containing citrate buffer (pH 6) for 12 min in three divided cycles. Detection system used was Envision (Dako). Di-aminobenzedine (DAB) was used for labeling and samples were counterstained lightly with haematoxylene.

The controls included positive control and normal control: (1) CD20-positive non-maltomatous high grade diffuse lymphoma of jejunum served as positive control; and (2) normal intestinal mucosa from margin of surgically resected small intestine served as normal control.

Immunostainings were interpreted with respect to positively stained plasma cells and lymphocytes in percentages of the total cells counted. Mean values were calculated. Light chain over-expression was documented when more than 75% of plasma cells and lymphocytes showed positivity for one of the light chains.

Informed consent was obtained from all patients before subjecting them to endoscopic procedure. Ethic Committee of the Institute approved the protocol.

Statistical analysis

Chi-square test and paired Student's *t* test were used for comparison. *P* value less than 0.05 was considered statistically significant.

RESULTS

The case collection ranged over thirteen years period. The clinical details are depicted in Table 1. Besides anemia, 1 patient had low platelet count (37 000 /mL). Urinary D-xylose excretion test (within 5 h after 5 g of oral D-xylose administration) was abnormal in 10 patients (normal > 1 g). Nine had increased fecal fat (7.67-30 g in 24 h, normal < 6 g/24 h). Abnormal D-xylose excretion test was recorded in 3 stage A and 7 stage B IPSID patients. Fecal fat level was increased in 3 and 4 patients with stage A and stage B IPSID, respectively. Stool examination for ova, cyst and parasite were negative in all patients. All cases had increased level for serum IgA. Pattern of intestinal involvement in radiology and endoscopy are shown in Table 1. Diagnosis of IPSID was based on demonstration of alpha heavy chain in serum and increased IgA levels (range, -340 to 660 mg%). Histology of all the 11 cases showed heavy plasma cell infiltration of the lamina propria admixed with variable amount of lymphocytes and scattered eosinophils. As a result of heavy mucosal cellular infiltration, increased mucosal thickness and wide spacing of the glands were observed (Figure 1). The mucosal infiltrating cell showed extension to sub-mucosa in 7 patients, and scattered

Table 1 Clinical parameters, histological staging, treatment profiles and disease outcome

Parameters	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th
Sex	M	M	M	M	M	M	F	M	M	F	M
Age (yr)	32	20	48	28	51	45	48	21	34	27	39
Clinical features											
Diarrhea	+	+	+	+	-	+	+	+	+	+	+
Weight loss	+	+	+	+	-	+	+	+	+	+	+
Pain abd	+	+	-	+	+	+	-	+	+	-	+
Clubbing	+	-	+	+	+	-	+	+	-	-	+
Anemia	+	+	-	-	+	-	+	+	+	+	-
Fever	+	-	-	-	+	+	-	-	-	+	-
Endoscopy											
Stomach	-	-	-	+ Infl	-	-	-	-	-	+ N	-
Duodenum	+	+	+(U)	+	+	+(U)	-	+	+(U)	+(U)	+
Jejunum	+	+	+	+	+	+	+	+	+	+(U)	+
Ileum	-	-	-	-	-	-	+	-	+	+	-
Colon	-	-	-	-	-	-	-	-	-	+	-
Radiology											
Mesenteric LN	-	+	+	+	-	+	-	+	+	+	-
Hepatomegaly	-	-	-	-	-	-	-	-	+	+	-
Splenomegaly	-	-	-	-	-	-	-	-	-	+	-
Histology (S+D+J+C+R)											
Number of tissue	4+4+0+0+0	4+4+0+0+0	5+3+0+0+0	2+4+4+0+0	3+4+0+0+0	4+5+0+0+0	4+5+0+0+0	3+5+0+0+0	3+4+3+0+0	2+3+3+3+4	4+4+0+0+0
Galian's stage	A	A	A	A, HP	B	B	B	B	B	B	B
Light chain over-expression	λ	κ	κ	-ive	-ive	-ive	κ	-ive	κ	κ	κ
Drugs received	Dox	Dox Chm	Dox Chm	Anti-HP	Dox	Dox	Dox Chm	Dox	Dox Chm	Dox Chm	Dox
Disease relapse	-	+	+	-	-	-	+	-	+	+	-
Follow-up (mo)	120	36	29	40	165	93	No FU	46	No FU	No FU	57

Abbreviations: Infl = inflammation, N = nodular, U = ulceration, + = disease present, - = disease absent, HP = *H pylori*, Dox = doxycycline, Chm = Chemotherapy (CHOP regime), Anti-HP = anti-*H pylori*, S = stomach, D = duodenum, J = jejunum, C = colon, R = rectum.

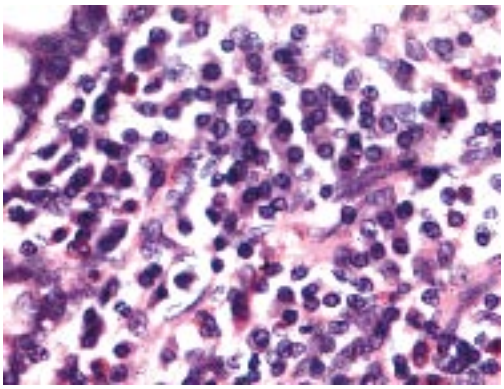


Figure 1 Photomicrograph of mucosal biopsy showing sheets of plasma cells infiltrating the lamina propria. Epithelial cell lining the glands are well preserved (HE, × 240).

immature plasma cells were also observed in 5 cases. One patient had histological evidence of active gastritis with *H pylori* in the gastric antral biopsy. In remaining cases, antral biopsies showed mild chronic inflammation of superficial mucosa and were negative for *H pylori*. Histologically (Galian's staging), stage A was found in 4 cases (1 case with *H pylori* infection) and stage B in 7 cases. Bone marrow was examined in 7 patients of stage B, but no atypical cells were detected in any biopsies.

Table 2 Immunoprofiles of the groups studied (mean ± SD)

Immuno-markers	Stage A	Stage B	Lymphoma	Normal
Syndecan-1	69 ± 3.40	51 ± 10.25	10.1 ± 6.354	11 ± 7.605
bcl6	1.75 ± 1.63	3.64 ± 3.541	4.25 ± 4.152	0.55 ± 0.51
p53	3.15 ± 3.257	6.5 ± 2.914	9.2 ± 4.315	0.11 ± 0.901
κ light chain	67.51 ± 16.952	67.42 ± 18.95	55 ± 2.399	42 ± 13.475
λ light chain	35 ± 13.857	24 ± 1.346	25.5 ± 1.087	29 ± 12.356
IgA heavy chain	72 ± 4.570	62 ± 24.521	22 ± 2.972	36 ± 6.513

Immunohistochemistry

Total number of positively stained cells was expressed in percentages and a total of 1000 cells were counted for each biopsy (Table 2).

Syndecan-1 expression: As shown in Figure 2, plasma cells had strong cytoplasmic positivity. Epithelial, endothelial and stromal cells showed faint cytoplasmic positivity. Only the plasma cells were counted and expressed in percentages. The mean values were significantly higher in IPSID patients compared to normal and lymphoma controls ($P < 0.05$). Stage A cases though had higher expression of syndecan-1 compared to stage B IPSID, the difference did not reach statistical significance.

bcl6 expression: As shown in Figure 3, bcl6 expression

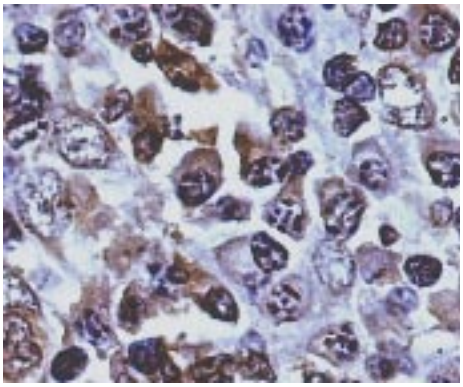


Figure 2 Photomicrograph showing strong syndecan-1-positive cells (PAP, × 440).

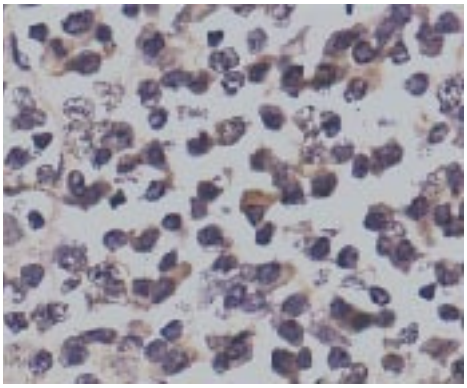


Figure 3 Photomicrograph showing bcl6-positive cells (PAP, × 440).

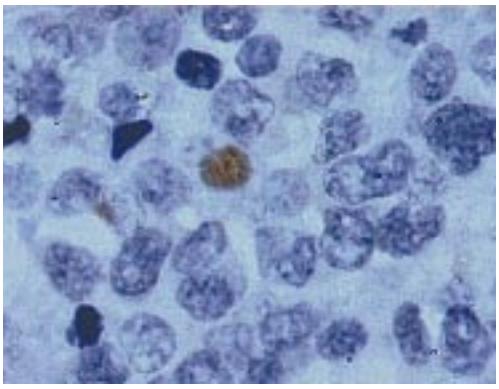


Figure 4 Photomicrograph showing p53 positivity in nuclei (PAP, × 1000).

was low on the whole; higher number of positively stained cells were seen in stage B IPSID and lymphoma control than stage A IPSID and normal control. Four of normal controls failed to stain. The differences between the mean values were not significant.

p53 expression: As shown in Figure 4, p53 expression was low and comparable amongst the different groups. Lymphoma had maximum number of positive cells, while lowest or negligible positive cells were observed in normal control. Stage A IPSID and normal control showed significantly lower mean values compared to lymphoma control ($P < 0.05$). However, no significant difference was observed between stage A, stage B and normal control.

Light chains

κ light chain: The expression of κ light chain was seen both in plasma cell and lymphocyte. Highest value was detected in stage A patients ranging from 12% to 80%; two patients had 78% and 80%, respectively, one patient had 12%, and the 4th patient had 58%. Stage B patients had the values ranging from 46% to 87%, and 4 patients showed more than 75%. Lymphoma had a range of 18% to 82% and 5 had more than 75%. Normal control had a range of 39% to 53%. Stage A and B IPSID patients had significantly higher values compared to normal and lymphoma controls ($P < 0.05$). κ light chain over-expression (> 75%) was seen in 2 of stage A and 4 of stage B patients. Few lymphocytes and plasma cells failed to stain.

λ light chain: The expression of λ light chain was found to be inversely correlated with κ light chain expression. There was a wide range for λ light chain expression. One stage A patient with λ light chain over-expression had a value of 78%. Those patients with κ light chain over-expression had values less than 25%. Two of stage A patients with κ light chain over-expression had 9% and 15% for λ light chain. The 4th stage A patient had 38%. Stage B patients had values ranging from 9% to 40%. The four patients with κ light chain over-expression had 9%, 15%, 20% and 18% of λ light chain, respectively. In the rest 3 patients, no over-expression of any of the two light chains had 30%, 36% to 40% for λ light chain. Lymphoma control had a range of 12% to 29% and one had 69%. Normal control had a range of 26% to 37%. Seven IPSID patients (3 stage A and 4 stage B patients) showed light chain over-expression (i.e. κ light chain in 6 and λ light chain in 1).

Heavy chain

IgA heavy chain expression was higher in IPSID patients compared to other groups. Stage A patients had values ranging from 58% to 81% and stage B had 40% to 91%. No significant differences were observed between stage A and B IPSID patients. Similarly, no significant differences were found between the patients of stage A and B with light chain over-expression and without light chain over-expression. Lymphoma control had values ranging from 13% to 30% and 5 of the control cases failed to express heavy chain. Normal control had values ranging from 32% to 45%. Stage A and B patients had significantly higher values than the two controls ($P < 0.05$).

Treatment and outcome

All cases were treated either with doxycycline (100 mg BD for 4 wk, followed by 100 mg OD for the next 6 to 12 mo) or tetracycline (500 mg TDS for 6 to 12 mo). Three patients initially received anti-tubercular drugs for a month with no clinical response. Subsequently, all three were diagnosed as IPSID (1 stage A and 2 stage B) based on demonstration of serum alpha heavy chain and heavy plasma cell infiltration in the endoscopic biopsies.

Stage A IPSID: The patient with λ light chain over-expression received 8 mo of doxycycline and remained disease-free at 120 mo of follow-up. Two patients with κ light chain over-expression responded initially to 9

mo of doxycycline and developed sub-acute intestinal obstruction at 18 and 11 mo of follow-up. Laparotomy revealed patches of proximal jejunal loops thickening. Resected intestinal segments showed trans-mural plasma cell infiltration mixed with lymphocyte and scattered immature plasma cell. Both patients received CHOP regime for six cycles and remained disease-free at 36 and 29 mo of follow-up, respectively. One patient with duodenal and proximal jejunal involvement had associated *H pylori*-positive active gastritis. Following treatment for *H pylori* (Omeprazole 20 mg BD, amoxycilin 500 mg BD and clarithromycin 250 mg TDS for 2 wk), he showed marked clinical improvement. Two weeks latter, repeat radiology and endoscopy showed no residual disease and no evidence of gastritis on repeat follow-up biopsy. He remained disease-free at 40 mo of follow-up. This particular patient did not have positivity for bcl6. The other 2 stage A patients failed show positive staining for bcl6. The 2 patients who developed frank lymphoma on follow-up had higher p53 and low syndecan-1 expression.

Stage B IPSID: Three patients received doxycycline for 9, 9 and 12 mo, respectively; all remained disease-free at 165, 93 and 46 mo of follow-up, respectively. One patient with κ light chain over-expression with hepatosplenomegaly and involvement of splenic flexure and stomach responded to 6 courses of CHOP and remained in remission for 6 mo. She went into relapsed with no response to treatment. At autopsy, terminal part of small intestine and proximal half of colon showed diffuse thickening with mucosal nodularity. Histology showed features of immunoblastic lymphoma with hepatic and splenic infiltration. One case with κ light chain over-expression was disease-free at 57 mo of follow-up. Three patients with κ light chain over-expression responded to doxycycline therapy and remained in remission at 35, 42, and 57 mo of follow-up. The first two patients had recurrence of the symptoms after 35 and 42 mo and biopsies from proximal jejunum revealed high-grade lymphoma. Subsequently, both were put on CHOP regime but failed to respond and died of the disease. Syndecan-1 expression was higher in four patients (57%, 61%, 65%, and 69%) and all remained disease-free at follow-up compared to the patients who had disease recurrence or died of the disease (36%, 40%, and 42%) ($P < 0.05$). Those 3 patients with unfavorable prognosis also had higher values for bcl6 but the differences were not significant ($P > 0.05$) (8%, 7%, and 5%) than those in remission (0.5%, 2%, 3%, and 3%). Expression of p53 was also obviously higher in those who succumbed to their illness (i.e. 11.5%, 9%, and 7%) than the ones who were in remission (1%, 4%, 5%, and 6%) ($P < 0.05$).

DISCUSSION

Syndecan-1 expression was found to be higher in all cases of IPSID compared to other groups. This would be in relation to number of plasma cell present. Higher mean values observed in stage A than stage B would suggest a phenotypic alteration of the cells i.e. benign to malignant clone. Syndecan-1-positive cell was significantly lower in lymphoma control cases ($P < 0.01$). Patients of stage A and B IPSID with unfavorable disease outcome also had low syndecan-1 expression compared to the ones with favorable outcome ($P > 0.05$). A higher syndecan-1 expression (> 50% positive) would infer a better prognosis. Similarly, such distinctive difference in the number of positive cell has been recorded in certain other types of tumors[26-30]. bcl6 expression was weak and low. Stage B IPSID patients and control lymphoma had comparatively higher values than stage A IPSID patients and other groups ($P > 0.05$). These three stage B cases subsequently developed high grade tumor had higher bcl6 expression. In our study, bcl6 appears to be a poor prognostic indicator. Similar observations have been made in diffuse large and marginal zone B-cell lymphomas and nodular variant of Hodgkin's lymphoma[31,34-36]. Besides, bcl6 expression would infer IPSID cells originating from germinal center B cell. Ariatti *et al*[33] made such suggestion with respect to bcl6-positive lymphoma. Mutant form of p53 is over-expressed by many malignant tumors including lymphomas. Similar to bcl6 expression, higher p53 values were recorded in those cases with poor outcome, whereas lower value was seen in those cases who were in remission at follow-up ($P < 0.05$). Minimum value was expressed by normal control followed by stage A and B IPSID and highest by lymphoma control.

Over-expression of one type of light chain was exhibited by our cases, as had been observed in few other studies[19-21,23]. Number of cases with κ light chain over-expression was more than that of λ light chain. Over-expression of λ light chain had also been reported in one case by Isaacson *et al*[19,20]. Monoclonality, exhibited by our patients, further strengthens the view expressed in previous studies, as an inherent character of IPSID as in lymphoma. However, monoclonality appeared not to influence the ultimate outcome of disease process. But study in more number of cases would be required before drawing such conclusion. IgA heavy chain over-expression was diffuse and uniformly strong in IPSID cases. This character of IPSID could be interpreted with important clinical implication. And this would help to identify and differentiate patients of IPSID, especially stage A, from other similar clinical conditions and investigative findings including histology.

Similar to our cases of *H pylori*-positive stage A IPSID, Fishbach *et al*[44] reported one patient in whom the underlying disease was controlled following eradication of *H pylori*. As stage A IPSID could be successfully treated with antibiotics, this effect of anti-*H pylori* could be more generalized rather than specifically related to *H pylori* eradication. Exposure to a long-standing cause and effect relationship between *H pylori* infection and IPSID localizing to foregut requires critical analysis and documentation in more number of cases. Gastric biopsy in the rest of cases did not show evidence of *H pylori* infection, though 1 case had evidence of infiltration by underlying disease process.

Colonic and hepatosplenic involvement in IPSID is usually seen only with overt lymphomatous conversion or in recurrent disease[47-50]. Three of our patients had gastric and colonic involvement at presentation with no overt lymphomatous conversion. But within one-year period, all the three cases developed overt lymphoma and two of the

cases succumbed to their illness in spite of the treatment

In conclusion, over-expression of syndecan-1, IgA and light chain would help in differentiating stage A IPSID from normal and stage B IPSID. Expression of bcl6 and p53 would indicate an aggressive behavior and a more advanced disease stage. bcl6 and p53 exhibited an inverse relationship with syndecan-1 expression. Gastric *H pylori* infection, though was seen only in one case with the active gastritis, would be advisable to look for in all cases of IPSID by carrying out gastric or pyloric antral biopsies.

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RAPID COMMUNICATION

Expression of E-selectin, integrin β1 and immunoglobulin superfamily member in human gastric carcinoma cells and its clinicopathologic significance

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Abstract

AIM: To study the expression levels of E- selectin, integrin β1 and immunoglobulin supperfamily member-intercellular adhesion molecule-1 (ICAM-1) in human gastric carcinoma cells, and to explore the relationship between these three kinds of cell adhesion molecules and gastric carcinoma.

METHODS: The serum contents of E-selectin, integrin β1 and ICAM-1 were detected by enzyme-linked immunosorbent assay (ELISA), in 47 healthy individuals (control group) and in 57 patients with gastric carcinoma (gastric carcinoma group) respectively prior to operation and 7 d after operation.

RESULTS: The serum E-selectin, ECAM-1 and integrin β1 were found to be expressed in both control and gastric carcinoma groups. However, they were highly expressed in patients with gastric carcinoma patients before operation or with unresectable tumours. The expression levels of ICAM-1 and integrin β1 were significantly higher in gastric carcinoma patients than in controls ($P < 0.01$). A comparison of the E-selectin levels between the two groups showed statistically insignificant difference ($P = 0.64$). In addition, the expression levels were all decreased substantially in the postoperative patients subjected to radical resection of the tumours, indicating that the high level expressions of these compounds might be the important factor for predicting the prognosis of these patients.

CONCLUSION: Serum E-selectin, ICAM-1 and integrin β1 expression levels are probably related to the metastasis and relapse of gastric cancer.

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Key words: Human gastric carcinoma cells; E-selectin; Integrin β1; ICAM-1; ELISA

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INTRODUCTION

The adhesion between endothelial cells and cancer cells is the essential intermediate link for tumor metastasis mediated by specific cellular surface receptors including selectin, integrin and cell adhesion molecule family[1]. Many investigations indicate that adhesion family members are involved in the progression of cancer[2]. The binding of cancer cells to endothelial cells mediated by the E- selectin is reportedly related to their metastatic potentials[3]. The intercellular adhesion molecule-1 (ICAM-1) a member of the immunoglobulin superfamily, its ligand is the adhesion molecule (LFA-1) of the immunoglobulin superfamily. Integrin-β1 is a member of the adhesion superfamily, which not only mediates the adhesion between cells and extracellular matrix, but also mediates the adhesion between leucocytes and blood vessel endothelial cells. The expression of E-selectin, ICAM-1 and integrin β1 on small blood vessels in tumour-infiltrating area at a high level is capable of suggesting the interaction between endothelial cells[4]. In the present study, we detected the serum E- selectin, ICAM-1 and integrin β1 contents in patients with gastric carcinoma in order to evaluate their clinicopathologic significance.

MATERIALS AND METHODS

Patients and healthy individuals

A total of 57 patients with gastric carcinoma, including 31 males and 26 females, at the age of 34-77 years , with a median age of 55 years, in Zhejiang Provincial People's Hospital and First People's Hospital of Taizhou were diagnosed as gastric carcinoma by pathological examination from January 2000 to December 2003. According to the WHO staging standard, 13 patients were classified into stage I, 5 patients stage II, 31 patients stage III, and 8 patients stage IV. These patients could also be

classified into intestine type and diffuse type based on Lauren's histological typing principle, and classified into well-, moderately- and poorly- differentiated types based on the predominant differentiation mode, respectively. All patients did not receive radiotherapy, chemotherapy, blood transfusion, steroid and opium drugs prior to operation. All the patients were followed up for 9 mo (1-48 mo) and their dates and causes of death were recorded.

Forty-seven healthy individuals, consisting of 31 males and 16 females, (at the age of 32-68 years, with a medium age of 51), were recruited as the control group. They were selected as blood donors and did not have any disease.

Assay procedure

The healthy individuals and patients were all fasted overnight. Blood samples were collected from their peripheral veins at 8-9 h AM next day, and then the blood samples were stored at -80℃ after the sera were separated by centrifugation. The blood samples from the patients with gastric carcinoma were collected both before and 7 d after operation respectively.

Assays for serum E-selectin, ICAM-1 and integrin β_1 were carried out using the solid phase ELISA test kit provided by the Parameter R &D Systems (USA), following the manufacturer's instructions. The test sensitivities were estimated to be 1 ng/mL, 2 ng/mL and 0.3 ng/mL for E-selectin, integrin β_1 and ICAM-1, respectively. Any resulting values for the patients which were 95% higher than those for the control were defined as the elevated contents of serum adhesion molecules. The cut-off values of E-selectin, ICAM-1 and integrin β_1 were 50.4 ng/mL, 337 ng/mL and 5.2 μg/mL, respectively. Therefore, if the cut-off values were higher than the above values, the high level expression should be considered as positive.

Statistical anaiysis

All data were analyzed by the SPSS 10.0 statistical software package, and their abnormal distributions were distinguished from the normal ones. At the same time, the variability of the medium values and the distributive ranges for the experimental data were evaluated using the unvariate analysis. The non-matched and matched data were evaluated using the variate Kruskal-Wallis analysis (analysis of variance, ANOVA), Mann-Whitney *U* test and Wilcoxon grade-related test. The multivariate analysis was conducted using Cox Proportional Hazards Regression Mode after the predicted variability was determined using the single factor analysis.

RESULTS

Contents of E-selectin, integrin β_1 and ICAM-1 and their comparison

The E-selectin, integrin β_1 and ICAM-1 were detected in all serum samples. The content of serum integrin β_1 in the preoperative gastric carcinoma group and control group was 4.8 μg/mL and 2.1 μg/mL, respectively (P = 0.00002). The content of ICAM-1 in the preoperative gastric carcinoma group and control group were estimated to be 271 ng/mL and 193 ng/mL, respectively. (P = 0.0004). The content of E-selectin in the preoperative gastric carcinoma group and control group was 42.1 ng/mL and 39.6 ng/mL, respectively (P = 0.64).

Table 1 Diagnostic value of elevated E- selectin, ICAM-1 and integrin β_1 levels in prediction of gastric carcinoma

Diagnostic value	E- selectin (> 50.4 ng/mL)	ICAM-1 (> 337 ng/mL)	Integrin β_1 (> 5.2 μg/mL)
Sensitivity (%)	24.6	33.3	28.1
Specificity (%)	95.7	95.7	95.7
Positive prediction value (%)	87.5	90.5	88.9
Negative prediction value (%)	51.3	54.2	52.3
Predominance ratio	7.32	11.25	8.78
95% confidence region	1.57-34.15	2.46-51.42	1.9-40.73

Expression levels of E-selectin, ICAM-1 and integrin β_1 in gastric carcinoma patients

The positive rates of E-selectin, ICAM-1 and integrin β_1 expression in gastric carcinoma group were 25.0%, 32.7% and 28.8%, respectively. The accuracy for diagnosing gastric carcinoma based on the expressions of these 3 adhesion molecules is shown in Table 1.

In gastric cacinoma group, 38 patients died in the progressive stage of gastric carcinoma, and tumours relapsed in 3 of the 19 remaining survivors. The serum E-selectin, ICAM-1 and integrin β_1 levels were higher in the progressive stage of tumour, and the elevated levels were significantly correlated with the tumour staging (P < 0.05).

No correlations were found between tumour T staging and serum E-selectin (P = 0.053) and ICAM-1 (P = 0.1)level. The serum E-selectin level [47.5 (36.8-58.3) ng/mL] in the patients with T4 tumour, was significantly higher than that [33.8 (28.4-41) ng/mL] of the patients with T_{1-3} tumour (P = 0.006) . Furthermore, the serum ICAM-1 level [292 (201-437) ng/mL] in the patients with T_3 and T_4 tumour-infiltrating serous membrane layer was significantly higher than that [231 (154-266) ng/mL] of the patients with T_1 and T_2 tumours in mucous membrane, submucous layer and proper muscular layer (P = 0.023).

The serum expression levels of E- selectin, ICAM-1 and integrin in patients with lymph node metastasis were higher than those in patients with no lymph node metastasis (the former *vs* the latter: P = 0.04, 0.004, and 0.0018, respectively). As compared with the non-remote metastatic group, the remote metastatic group had higher serum E-selectin, ICAM-1 and integrin β_1 levels (P < 0.05). The serum E-seletin, ICAM-1 and integrin β_1 levels were not correlated with the tumour location (gastric antrum, gastric body or gastric cardia), tumour typing and differentiation degree (Table 2).

Effect of surgery on expression of E-selectin, ICAM-1 and integrin β_1

Among the 57 patients with gastric carcinoma, 41 were subjected to radical gastric resection and regiongal lymph node cleaning, 16 had unresectable tumours. All the patients had no postoperative complications. The postoperative serum E-selectin, ICAM-1 and integrin

Table 2 Variability analysis of the prognoses and predictive factors in patients with gastric carcinoma

Variables	Risk ratio	95% confidence region	*P*
Age	0.97	0.94-1.01	0.2
Sex	0.77	0.39-1.54	0.47
Tumor location (gastric antrum, gastric body or gastric cardia)	0.95	0.62-1.45	0.81
Tumour histology (intestine type and diffusion type)	1.32	0.70-2.52	0.38
Tymour differentiaton degree (high,moderate, low)	1.19	0.71-1.96	0.49
Tumour staging (stages I-IV)	2.65	1.71-4.11	0.000
T (T_1-4)	2.75	1.75-4.34	0.000
N (No, N_1)	4.33	1.65-11.36	0.003
M (Mo, M_1)	7.36	2.98-18.2	0.000
E-selectin (elevated, normal)	2.81	1.40-5.63	0.003
ICAM-1 (elevated, normal)	2.71	1.37-5.36	0.004
Integrin β_1 (elevated, normal)	2.1	1.05-4.18	0.035

β_1 contents decreased significantly (P = 0.04, 0.01, and 0.001, respectively). Conversely, the postoperative serum E-selectin, ICAM-1 and integrin β_1 levels in the16 patients with nonresectable tumours were similar to those before operation (P = 0.08, 0.09, and 0.2, respectively).

Variability analysis of prognosis and predictive factors in patients with gastric carcinoma

The monovariate analysis revealed that the TNM staging, tumour-infiltrating deepth in gastroparietes (T status), lymph node metastasis, remote metastasis, as well as the preoperative serum E-selectin, ICAM-1 and integrin β_1 levels were the important factors affecting the total survival rate of the patients (Table 2). The multiple statistical analysis of all the factors was carried out, the results indicated that tumour staging was the only independent factor for predicting the survival of the patients.

DISCUSSION

The present study demonstrated that the preoperative high levels of ICAM-1, integrin β_1 and E-selectin in patients with gastric carcinoma had higher specificity and lower sensitivity for the diagnosis of gastric carcinoma. The serum contents of the three kinds of adhesion molecules significantly correlated with tumour staging, gastropariete infiltrating, lymph node metastasis and remote metastasis. Although the relationship between serum ICAM-1 and metastasis in the present study further proved the previous observations, the correlation between the serum E-selectin and integrin β_1 contents and remote metastasis is reportedly contradictory sometimes[5-8].

Previous investigations showed that certain kinds of cytokines are able to induce the expression of E-selectin, ICAM-1 and integrin β_1[9,10]. Monocytes are the only origin of sICAM-1. Large amounts of sICAM-1 in serum could be found in the culture of certain tumour cell strains' indicating that tumour cells may also be the origin of sICAM-1 which may explain the decreased serum sICAM-1 in patients with gastric carcinoma after resecting of their tumours. Moreover, sICAM-1 could not be produced through different forms of mRNA splicing[8,10].

In the present study, all the preoperative serum E-selectin, ICAM-1 and integrin β_1 levels in the patients with gastric carcinoma were found to be an important factor affecting the prognosis of the patients, suggesting that the levels of E-selectin, ICA-1 and integrin β_1 can be used as supplementary markers to determine the disease condition, stage and prognosis, as well as the therapeutical efficacy in the patients. However, multiple statistical analyses of all the factors have revealed that tumor staging could be used to predict the survival of cancer patients as an independent factor. The discrepancy between effects of the adhesion molecule levels in peripheral circulation on the prognosis of patients with gastric carcinoma in different reports is possibly attributed to the different number of patients used and the different progression stage of the disease[9-11].

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CASE REPORT

Peliosis hepatis as an early histological finding in idiopathic portal hypertension: A case report

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Abstract

Peliosis hepatis is a rare condition characterized by dilatation of hepatic sinusoids and blood-filled spaces in the liver mainly observed in subjects exposed to toxic substances or estrogens, which is frequently asymptomatic. Non-cirrhotic idiopathic portal hypertension (NCIPH) is also a vascular disease of the liver rarely observed in European countries, which is usually diagnosed only when the hemorrhagic complications of portal hypertension occur. We report a case of NCIPH in a young Caucasian male who was diagnosed with liver peliosis, showing ultrasonographic and endoscopic signs of portal hypertension four years after. A second biopsy was diagnostic for NCIPH. Even if the pathogenesis remains obscure, peliosis hepatis can be considered as an early sign of vascular disease of the liver, which may progress to more definite conditions.

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Key words: Peliosis hepatis; Idiopathic portal hypertension; Hepatic veins catheterisation

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INTRODUCTION

Portal hypertension in Europe is mainly due to intra-hepatic causes, almost exclusively by liver cirrhosis, which leads to an increase in sinusoidal pressure. While the identification of pre-hepatic causes such as extra-hepatic portal vein thrombosis is now feasible by non invasive means such as eco-color-Doppler and CT-scan, rarer forms of intra-hepatic pre-sinusoidal portal hypertension such as non cirrhotic idiopathic portal hypertension (NCIPH) are difficult, and rely on invasive techniques such as hepatic vein catheterisation and hepatic biopsy. We describe a case of NCIPH developed in a young Italian man who was first diagnosed as peliosis hepatis carrier. Hepatic vein catheterisation confirmed the clinical suspicion, and the diagnosis was finally ascertained by histology. This is the first reported case in which NCIPH follows a peliosis hepatis. This may represent an early histological finding in case of vascular diseases of the liver.

CASE REPORT

A 36-year-old Italian man was referred to our unit for a sonographic evaluation of chronic liver disease. He was in very good clinical condition, did not drink alcohol, and was neither exposed to toxic substances nor to any drugs. The patient also denied any exposure to anabolic steroids. Family history indicated a grandmother died of digestive bleeding in the setting of end-stage primary biliary cirrhosis (PBC). Relevant findings in patient's history were a persistent slight elevation of gamma-glutamyl-transferases and aminotransferases associated with leuco- and thrombocytopenia noticed for the first time at the age of 26 years. At the age of 28 years, the patient suffered from spontaneous bilateral sublaxation of the shoulder, for which he underwent surgical repair. A muscle biopsy taken during the intervention was normal. Due to the persistence of abnormal laboratory findings mentioned above, at 32 year-old he was examined in another hospital and screened for chronic liver disease. Laboratory tests excluded viral, metabolic and autoimmune causes of liver disease. Abdominal ultrasound scan showed only a slight enlargement of the spleen (longitudinal diameter 130 mm), while liver and portal system were normal, no sign of portal hypertension was evident. A liver biopsy was performed, showing the typical aspects of microscopic peliosis. The patient also underwent a bone marrow

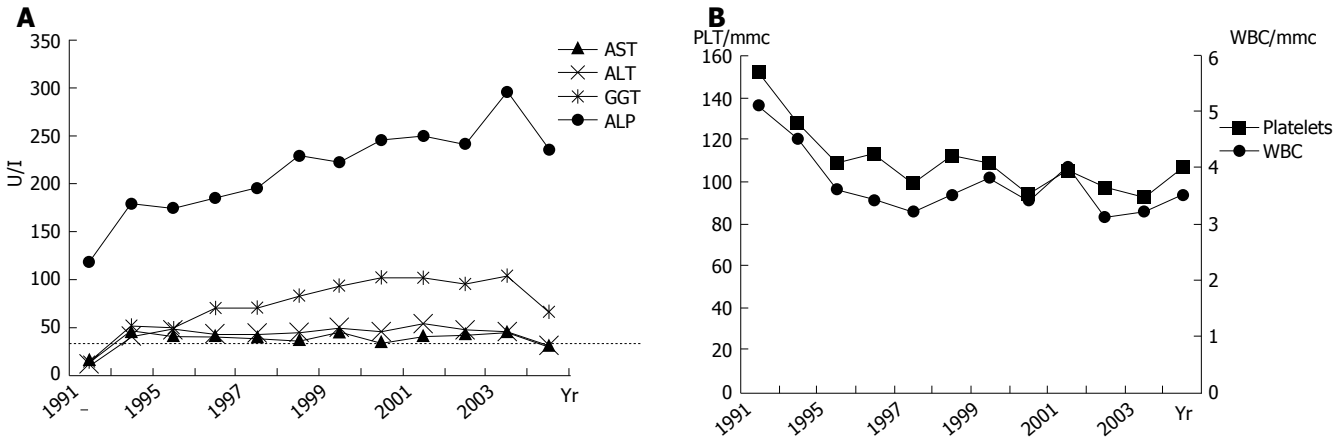


Figure 1 Aminotransferases and cholestasis enzymes (**A**) and white blood cells (WBC) and platelets (**B**) during follow-up.

Table 1 Laboratory findings on admission and after ursodeoxycholic acid (UDCA)

	On admission	During UDCA treatment (one year)
Haemoglobin (g/L)	146	148
White cells (× 10^3/mL)	3.12	3.5
Neutrophils (%)	59.2	60.3
Platelets (× 10^3/mL)	93	107
Creatinine (mg/dL)	1.08	0.9
Na^+(mEq/L)	143	140
K^+ (mEq/L)	3.9	4.1
AST (IU/L)	44	31
ALT (IU/L)	47	31
ALP (IU/L)	241	235
GGT (IU/L)	95	67
CPK (IU/L)	182	160
LDH (IU/L)	284	225
INR	1.03	1.11
Albumin (g/L)	4.3	4.1
Bilirubin (mg/dL)	1.04	0.6
Cholesterol (mg/dL)	212	228
Triglycerides (mg/dL)	81	89

biopsy in the same year, due to persistent leuco- and thrombocytopenia, which revealed a normal pattern.

The Echo-color-Doppler performed at our unit demonstrated a coarse liver echo-texture, splenomegaly (longitudinal diameter 155 mm), patency of portal vein and intrahepatic branches, with normal portal blood flow velocity (15 cm/s, normal value > 14), patency of splenic vein and superior mesenteric vein, and US signs of portal hypertension, namely ectasia of left gastric vein with hepatofugal flow and a small spontaneous spleno-renal shunt. Intraparenchymal branches of hepatic and splenic artery showed high resistance and pulsatility indices. Hepatic veins were patent and showed a normal triphasic flow.

Since portal hypertension had not been detected before, the patient was hospitalized to perform a diagnostic procedure.

Physical examination demonstrated that the patient's stature was 1.65 m, his weight 70 Kg, arterial blood pressure was 115/70 mmHg, and heart rate was 48 bpm. He showed hypertrophy of proximal muscles of upper and lower limbs (the patient made no significant physical activity), multiple small telangiectasias on the trunk and lower limbs, splenomegaly, and white striae on the flanks.

Figure 1 shows laboratory findings in patient's history, and Table 1 lists those at the moment of first visit at our unit. Alanine aminotransferasis (ALT) and gamma-glutamyl transferases (GGT) were persistently elevated during the last 10 years, but no sign of liver failure was present. Autoantibodies for PBC, primary sclerosing cholangitis, connective tissue diseases and autoimmune thyroiditis tested were negative. Inherited thrombogenic disorders and hematologic diseases were also excluded. An electrocardiogram showed sinus bradycardia (48 bpm) but no other pathological aspects. An upper digestive tract endoscopy confirmed portal hypertension showing small esophageal varices. The patient also underwent an invasive hepatic hemodynamic study. By accessing *via* right brachial vein under fluoroscopic control, a 5F balloon-catheter was advanced in the main hepatic vein and hepatic venous pressure gradient (HVPG) was obtained by the difference between wedged (by inflating the balloon) and free pressures. Figure 2 shows tracing of hepatic vein pressures. Free pressure was normal, wedge pressure and HVPG were only slightly elevated (HVPG 7 mmHg, normal value 1-5 mmHg), suggesting idiopathic portal hypertension. A needle liver biopsy was then obtained. Liver tissue samples were examined by the same experienced pathologist (F.C.) who studied the first ones. Histology showed periportal fibrosis, obliteration of small portal veins and some areas of nodular regenerative hyperplasia (NRH), which were consistent with the diagnosis of NCIPH. No alteration of bile ducts was noted nor cholestasis. Peliosis hepatis was no more observable.

Due to the slightly cholestatic feature of liver disease, we empirically treated the patient with a high dosage (15 mg/Kg of body weight) of ursodeoxycholic acid (UDCA). After one year of treatment GGT decreased to near-normal levels and aminotransferases became normal (Table 1 and Figure 1A).

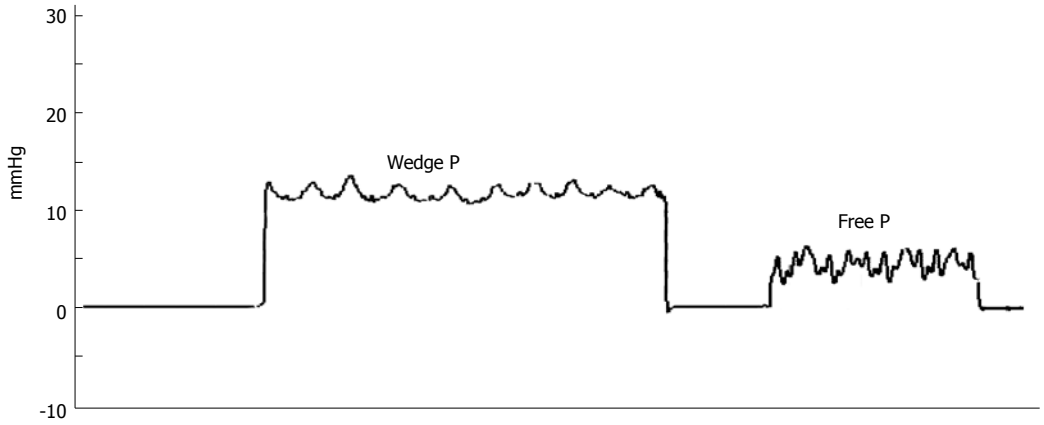


Figure 2 Hepatic hemodynamic findings consistent with the diagnosis (wedge pressure 12 mmHg, free pressure 5 mmHg, hepatic venous pressure gradient 7 mmHg).

DISCUSSION

Peliosis hepatis is a rare disorder characterized by the presence of cystic, blood-filled spaces of variable size in the liver[1]. Endothelial cells cover the internal surface of these cavities, and adjacent hepatocytes can be normal or show atrophic or degenerative changes[2]. A microscopic form and a macroscopic form have been described. In the microscopic form ultrasound and CT do not show any macroscopic change in the liver. It has been reported mainly in adult patients in association with chronic diseases (AIDS, malnutrition, tuberculosis, lepropsy, vasculitis, haematological neoplasias, hepatocellular adenoma and hepatocarcinoma), various pharmacological agents (androgenic anabolic steroids and estrogens, immunosuppressive drugs, tamoxifene), toxic substances (arsenic, vinyl chloride), infections (Bartonella henselae)[3], and in renal transplant recipients receiving immunosuppressive treatment[4,5]. The morphogenesis of peliosis is controversial. It has been attributed to an increased sinusoidal pressure because of difficulties in blood outflow from the liver, the disappearance of normal parenchyma by necrosis of liver cells, and sinusoid wall weakness. Since the disease is very rare, data about its natural history are scarce and clinical spectrum varies from asymptomatic cases to severe complications, such as hemoperitoneum[3].

Idiopathic non-cirrhotic intrahepatic portal hypertension (NCIPH) was first identified by Banti in 1898[6]. An increased portal pressure with patent portal and hepatic veins characterizes it, in the absence of cirrhosis[7]. The anatomic basis for these alterations is an increased fibrous component in portal vessel wall and periportal and perisinusoidal space, inducing a pre- and perisinusoidal resistance to flow of portal venous blood. Therefore wedge hepatic venous pressure does not reflect the increased pre-sinusoidal portal pressure and HVPG is usually normal or near-normal[8].

NCIPH is clinically characterized by the signs of portal hypertension (varices and portosystemic collateral vessels), overt splenomegaly with pancytopenia, and relatively mild abnormalities in liver function tests[9]. Bilirubin is reported to be normal in NCIPH series, but literature does not mention GGT levels. To our knowledge this is the first reported case in which GGT is elevated at presentation.

While NCIPH is quite common in India and Japan, it is rarely observed in developed countries[10,11]. The clinical course of the disease is usually silent until gastrointestinal bleeding secondary to variceal rupture develops. If variceal bleeding can be controlled, 5-year survival rate is about 95%[9].

The recognized causes are varied. Schistosomiasis[12] and toxic substances (arsenic, vitamin A) are the most frequent ones, interestingly the latter ones are the same agents recognized as causes of peliosis hepatis. In many cases the etiology remains unknown, and the disease is defined "idiopathic". In these cases immunological disturbance (autoimmune diseases such as scleroderma, mixed connective tissue disease, autoimmune thyroiditis), thrombophilic status, latent myeloproliferative disorders, infections and/or increased fibrogenesis in the portal tract are suspected as being candidates for the primary agents[13]. Hillaire and colleagues[14] have recently analysed a series of patients with NCIPH identifying a thrombophilic status (overt or latent) in the majority, but this could be excluded in the case we report.

As for biopsy features, many pathological entities such as idiopathic portal hypertension, benign intrahepatic portal hypertension, hepatoportal sclerosis, nodular regenerative hyperplasia, and incomplete septal cirrhosis can be grouped under the term idiopathic NCIPH, as they frequently show a number of overlapping features[14,15]. The finding of peliosis hepatis in the first biopsy of our patient suggests that this entity may represent a praecox presentation of the vascular changes, which occur in NCIPH. Our observation is supported by the paper of Cavalcanti and colleagues[4], who performed serial liver biopsies in a group of kidney-transplanted patients with peliosis hepatis, showing that the disease can evolve, leading to regenerative nodular hyperplasia, perisinusoidal fibrosis and cirrhosis in some cases.

Another interesting finding in our patient is a second grade relative affected by primary biliary cirrhosis (PBC). Among autoimmune diseases, PBC has been shown to have a strong heritability[16], and some familial cases describing the involvement of second-degree relatives have been reported[17,18]. PBC is a cause of non-cirrhotic portal hypertension in the first stage of the disease, and some cases of PBC resembling NCIPH have been previously reported[19]. Even if bile ducts were found to be normal in

our patient's biopsy and no clear evidence of an overlap could be proved, interestingly an empiric treatment with UDCA induced a normalization of aminotransferases and a reduction of GGT (Table 1).

When all the known causes of NCIPH are excluded the finding of previous bilateral glenohumeral instability, white striae at flanks, hypertrophy of proximal muscles of upper and lower limbs and peliosis hepatis in the same individual make conceivable to hypothesize that some unrecognized disturbances of connective tissue can be linked to all these conditions, eventually leading to NCIPH.

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LETTERS TO THE EDITOR

Noni juice is not hepatotoxic

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Abstract

Noni juice (*Morinda citrifolia*) has been approved for use as a safe food within the European Union, following a review of safety. Since approval, three cases of acute hepatitis in Austrian noni juice consumers have been published, where a causal link is suggested between the liver dysfunction and ingestion of anthraquinones from the plant. Measurements of liver function in a human clinical safety study of TAHITIAN NONI® Juice, as well as subacute and subchronic animal toxicity tests revealed no evidence of adverse liver effects at doses many times higher than those reported in the case studies. Additionally, *M. citrifolia* anthraquinones occur in the fruit in quantities too small to be of any toxicological significance. Further, these do not have chemical structures capable of being reduced to reactive anthrone radicals, which were implicated in previous cases of herbal hepototoxicity. The available data reveals no evidence of liver toxicity.

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Key words: Noni juice; *Morinda citrifolia*; Novel food; Human clinical safety study

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TO THE EDITOR

Morinda citrifolia (noni) fruit juice was approved as a novel food ingredient in pasteurized fruit drinks by the European Commission Decision of 05-06-2003. This approval was based on the opinion of the EU Scientific Committee on Food (SCF) of Tahitian Noni® Juice, following a safety review of this product[1].

During 2004, an Austrian case report was published in which it is suggested that noni fruit juice may be responsible for acute hepatitis[2]. A second report was published in the August 2005 issue of this journal in which noni juice was again suggested to be the cause of two other cases of acute hepatitis in Austria[3]. In both publications, the authors suggest the presence of anthraquinones in the juice may be responsible for the liver dysfunction observed in their patients. However, experimental data fails to reveal any direct liver toxicity from noni juice.

TAHITIAN NONI® Juice, the brand associated with the latest case reports, was investigated in a human clinical study (unpublished data. Mugglestone C *et al*. A single centre, double-blind, three dose level, parallel group, placebo controlled safety study with TAHITIAN NONI® Juice in healthy subjects, BIBRA International Ltd. UK, 2003). Ninety-six subjects were randomly assigned to four groups. These groups included a placebo group and three test groups, receiving up to a dose of 750 mL TAHITIAN NONI Juice per day. For 28 d, subjects drank 750 mL of either the placebo or juice containing one of three doses of TAHITIAN NONI Juice.

Several parameters were investigated with measurements being made at study screening, d 0 (baseline), wk 2, wk 4, and wk 6. The measurements most applicable to the evaluation of liver function and hepatocellular disease were alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (BIL), gamma-glutamyl transferase (GGT), total protein, and prothrombin time (which is useful for determining the extent of hepatocellular disease).

Other study measurements included hemoglobin, hematocrit, mean cell volume, red cell count, activated partial thrombin time, total and differential white cell count, platelet count, lipids (LDL, HDL, cholesterol, triglycerides), creatine kinase, creatinine, gamma-glutamyl transferase, glucose, total protein, and uric acid. Additionally, urinalysis involved semi-quantitative ("dipstick") analysis for leucocytes, nitrite, urobilinogen, protein, pH, blood, specific gravity, ketones, bilirubin and glucose. Where necessary, a urine cyto-bacteriological examination was performed to characterize or count crystals, casts, epithelial cells, white blood cells, red blood cells and bacteria. Vital signs were measured, including systolic and diastolic blood pressure and heart rate. ECG measurements (12 lead) were also made for each subject. All adverse events were recorded. Selected mean laboratory values for the various groups are presented in Table 1.

Differences between mean values were clinically insignificant and well within the range of normal values. Furthermore, there was no evidence of any dose-related adverse events. The study results indicate that TAHITIAN

Table 1 Mean clinical laboratory values by week and dose groups

Parameter	Value	Placebo	30 mLTNJ	30 mL TNJ	750 mLTNJ
ALP (U/L)	Mean wk 0	61.67	58.65	54.00	54.44
	Mean wk 2	55.80	57.83	51.16	54.47
	Mean wk 4	63.56	59.48	54.99	53.07
	Mean wk 6	62.69	61.50	58.22	55.04
ALT (U/L)	Mean wk 0	17.80	18.00	17.88	21.98
	Mean wk 2	18.53	19.53	17.80	24.11
	Mean wk 4	18.99	20.10	17.75	22.71
	Mean wk 6	19.21	16.90	17.63	20.92
AST (U/L)	Mean wk 0	18.30	18.55	18.27	20.71
	Mean wk 2	18.04	19.67	19.18	22.32
	Mean wk 4	18.93	19.55	19.19	20.90
	Mean wk 6	18.42	18.95	18.64	19.87
Total bilirubin	Mean wk 0	12.12	10.55	10.93	11.30
(μmol/L)	Mean wk 2	13.19	11.31	11.47	14.20
	Mean wk 4	12.23	11.63	12.07	14.03
	Mean wk 6	14.77	11.99	11.44	13.98
GGT (U/L)	Mean wk 0	20.31	24.74	22.51	21.36
	Mean wk 2	21.19	26.74	23.03	20.99
	Mean wk 4	21.87	26.54	21.14	21.21
	Mean wk 6	21.11	24.22	20.00	21.07

Table 2 Serum values in female Sprague Dawley rats in 1st 13-wk study (Mean ± SD)

Females					
Dose	ALT (μkat/L)	AST (μkat/L)	ALP (μkat/L)	BIL (μmol/L)	GGT (μkat/L)
Control	2.1 ± 0.5	2.26 ± 0.46	2.07 ± 0.27	1.21 ± 0.61	0.02 ± 0.01
0.4 mL/kg	1.89 ± 0.62	2.15 ± 0.81	2.22 ± 0.29	1.43 ± 0.52	0.01 ± 0.01
4 mL/kg	1.98 ± 0.34	1.99 ± 0.39	2.08 ± 0.41	1.36 ± 0.54	0.01 ± 0
8 mL/kg	1.94 ± 0.33	1.74 ± 0.18	2.3 ± 0.45	1.22 ± 0.63	0.01 ± 0

Table 3 Serum values in male Sprague Dawley rats in 1st 13-wk study (Mean ± SD)

Males					
Dose	ALT (μkat/L)	AST (μkat/L)	ALP (μkat/L)	BIL (μmol/L)	GGT (μkat/L)
Control	2.47 ± 0.42	2.11 ± 0.58	2.97 ± 0.33	1.59 ± 0.32	0.01 ± 0.01
04 mL/kg	2.27 ± 0.52	1.8 ± 0.56	2.82 ± 0.39	1.5 ± 0.41	0 ± 0
4 mL/kg	2.35 ± 0.44	1.86 ± 0.32	2.77 ± 0.36	1.5 ± 0.36	0 ± 0
8 mL/kg	2.4 ± 0.61	2.06 ± 0.76	3.18 ± 0.44	1.6 ± 0.49	0.01 ± 0

Table 4 Serum values in female Sprague Dawley rats in 2nd 13-wk study (Mean ± SD)

Females					
Dose	ALT (μkat/L)	AST (μkat/L)	ALP (μkat/L)	BIL (μmol/L)	GGT (μkat/L)
Control	2.01 ± 0.3	1.73 ± 0.43	2.24 ± 0.36	1.01 ± 0.77	0.01 ± 0.01
20 mL/kg	2.14 ± 0.52	1.99 ± 0.71	2.44 ± 0.47	1.17 ± 0.72	0.01 ± 0.01
50 mL/kg	1.91 ± 0.53	1.82 ± 0.43	2.14 ± 0.43	1.24 ± 0.58	0.01 ± 0.01
80 mL/kg	1.25 ± 0.25	1.22 ± 0.12	1.98 ± 0.22	1.62 ± 0.77	0.01 ± 0.01

Table 5 Serum values in male Sprague Dawley rats in 2nd 13-wk study (Mean ± SD)

Males					
Dose	ALT (μkat/L)	AST (μkat/L)	ALP (μkat/L)	BIL (μmol/L)	GGT (μkat/L)
Control	2 ± 0.26	1.83 ± 0.21	3.51 ± 0.42	1.23 ± 0.25	0 ± 0
20 mL/kg	1.95 ± 0.3	1.79 ± 0.31	3.09 ± 0.47	0.93 ± 0.56	0 ± 0
50 mL/kg	1.55 ± 0.2	1.53 ± 0.28	3.17 ± 0.38	1.27 ± 0.45	0 ± 0
80 mL/kg	1.67 ± 0.4	1.77 ± 0.47	3.27 ± 0.29	1.03 ± 0.45	0 ± 0

NONI® Juice is safe to consume, in quantities up to 750 mL/d, confirming the EU SCF opinion that high consumption quantities are appropriate.

The aqueous extract of *M. citrifolia* fruit was evaluated in a repeat dose oral toxicity assay in rats[4]. For 28 d, Sprague Dawley rats received 1000 mg/kg body weight. Animals were observed for clinical symptoms, body weight was recorded, and hematology and serum chemistry parameters were measured.

Among the serum chemistry measurements were AST, ALT, and GGT. There were no significant differences between the males and females of the test and control groups, with the exception of a lower AST value observed in the females of the treatment group, and an exceptionally low ALT value for the males of the control group that resulted in a statistical difference for treated males. In either instance, however, the values observed in the treatment groups were within the normal range for these animals and did not correspond to a dose-response relationship. These results did not reveal any adverse liver effects.

Two 13-wk oral toxicity studies of TAHITIAN NONI® Juice in rats were performed (unpublished data, Glerup P. TAHITIAN NONI® Juice-A 13-wk oral (gavage) toxicity study in rats. Scantox Biologisk Laboratorium, Lille Skensved, Denmark, Lab no. 35 207, March 2000; and Glerup P. TAHITIAN NONI® Juice-A 13-wk oral (gavage) toxicity study in rats. Scantox Biologisk Laboratorium, Lille Skensved, Denmark, Lab no. 39 978, May 2001). In each study, 80 Sprague Dawley rats (40 males and 40 females) were included. Six consecutively higher doses were examined in each study, with a high dose equivalent to 80 mL TAHITIAN NONI® Juice/kg per day (20 mL/kg of 4-fold concentrated juice).

Study measurements indicative of liver effects were ALT, AST, ALP, GGT, total bilirubin, total protein, protein by electrophoresis, globulins, albumin/globulin ratio, and prothrombin time. Additionally, clinical observations included liver weights, and macroscopic and microscopic (histological) examination of the livers. Laboratory data for liver function for each dose group in each study are mentioned in Tables 2, 3, 4, and 5.

No treatment-related changes were observed in any of the dose groups for any of these measurements, including histological examinations, demonstrating a lack of toxicity to the liver. Consequently, the No Observable Adverse Effect Level (NOAEL) was determined to be greater than 80 mL TAHITIAN NONI Juice/kg per day.

The NOAEL is much higher than the amounts ingested

by the patients described in the case studies. In the most recent publication, the first patient consumed a daily dose of 71.4 mL (1.5 L consumed over 3 wk). The second case reported in this publication describes a daily consumption rate of 23.8 mL (2 L consumed over 3 mo). The weight of each patient is not given, but assuming an average weight of 70 kg, the doses consumed would be 1.02 mL/kg per day and 0.34 mL/kg per day. The NOAEL of the animal studies is almost 80 times greater than these two doses. The high dose in the clinical study was approximately 10 times higher.

The first published case report claims that the patient drank a glass of noni juice every day for a few weeks. No exact quantity is specified; no indication is given as to how large the glass was or how much of it was filled. However, assuming a full cup volume of 250 mL and a patient weight of 70 kg, the dose would be 3.6 mL/kg. The NOAEL of the animal studies is 22 times greater than the ingestion rate of this patient, and the clinical study high dose is three times more. Not only are all reported doses in these cases well within the highest doses of the controlled studies, where no liver effects were observed, but were also sufficiently low to assume a large margin of safety. If there is any true causal link, it is likely to be only idiosyncratic in nature.

As a pretext for implicating noni juice in liver toxicity, the authors of both reports cite publications containing only limited information, including similar case reports for the herbal laxative drugs Senna (*Cassia augustifolia*)[5] and Cascara sagrada (*Rhamnus purshianus*)[6]. The laxative effects of these herbal preparations are due to large quantities of a specific class of anthraquinones[7] (different than those in the noni plant).

In these cases, the patients had ingested large quantities of the herbs for an extensive time. Consequently, the investigators propose a potential involvement of anthraquinones. A similar assumption is made in the few case reports of other herbs cited by the authors of these two publications[8-10]. In these instances, however, the role of the anthraquinones directly isolated from these herbs, and linked to the patients' liver dysfunction was not supported by any additional experimental evidence. In fact, support for these assumptions is based on limited information from experimental studies of sennosides[11] and luteoskyrin[12], which do not occur in the noni plant.

The suggestion of an anthraquinone involvement in the three Austrian cases is not based on solid data. The first argument against such a causal link is a quantitative one. Until very recently, investigators had been unable to identify or isolate anthraquinones from noni fruit[13]. However, two recent publications have reported the presence of anthraquinones in noni fruit, at very low concentrations. The first report describes 48.3 mg of isolated material, distributed amongst six anthraquinones, from 1.3 kg of dried noni fruit from Indonesia[14]. On a dry weight basis, the total concentration is 37×10^{-6}. In the juice form, the concentration would be approximately 3×10^{-6}. The second publication describes isolating a few micrograms of anthraquinones from 9 kg of dried fruit[15]. The anthraquinone concentration in this study was less than 1×10^{-6}. Such low yields and concentrations are responsible for the inability of earlier researchers to identify anthraquinones in the fruit.

In comparison to Senna and Cascara sagrada, the total anthraquinone content of noni fruit is insignificant. The European Pharmacopoeia describes Senna leaf as containing no less than 2.5% (25×10^{-3}) anthraquinones; the standardized extract is to contain no less than 5.5%. Cascara sagrada is described as having a minimum anthraquinone content of 8% (80×10^{-3}). These concentrations are many thousands of times higher than that in noni juice. Where a large quantity of Senna and Cascara is required to induce liver toxicity, it is unreasonable to assume a barely detectable quantity of anthraquinones in noni juice can have the same effect.

Beyond the minute quantity of anthraquinones in noni fruit juice is a second issue of chemical structure. Anthraquinones do not have the same universal biological effects. Differences in bioactivities, toxic or otherwise, are seen amongst variants of other types of compounds as well. Thus, it is not reasonable to assume that one category of anthraquinones will have the same exact toxic action as another. The anthraquinones present in the laxative herbs, linked to the previous cases of hepatotoxicity, are 1, 8-di hydroxyanthraquinones (1, 8-DHA's). On the other hand, those that occur in *M. citrifolia* are substituted 9, 10-anthraq uinones[16,17]. These structural differences result in different modes of action. The 9, 10-anthraquinones can not be reduced in biological systems to form anthrone radicals, while 1, 8-DHA's can[18]. It is the formation of anthrone radicals that is required to produce tissue damage[19].

In summary, human and animal studies of high doses of TAHITIAN NONI® Juice reveal no adverse liver effects. Further, anthraquinones in noni fruit are of insufficient quantities and fail to possess the necessary chemical structures to cause liver tissue damage. The available data demonstrates that noni juice is not directly toxic to the liver

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Meetings

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Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
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symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
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ILTS 12th Annual International Congress
3-6 May 2006
Milan
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www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
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7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
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convention@edinburgh.org
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
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www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
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EDITORIAL

Autoimmune liver disease and the Canadian First Nations Aboriginal Communities of British Columbia's Pacific Northwest

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Abstract

Primary biliary cirrhosis (PBC) is a well-known but uncommon chronic liver disease that is presumed to be of autoimmune etiology. Recently, investigations in British Columbia (BC), a province of Canada situated along the Pacific North-West of North America, have suggested that PBC is not a rare disease amongst BC's Aboriginal (i.e. First Nations) communities. Geographically, BC is adjacent to South East Alaska, an American state that has also reported an increased prevalence of PBC amongst its Aboriginal communities. In this article, the medical evidence supporting a hypothesis of increased risk of PBC amongst BC's First Nations communities is reviewed. Evidence suggesting that autoimmune hepatitis is also more likely amongst BC's First Nations communities is also presented.

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Key words: Primary biliary cirrhosis; Aboriginal; First Nations; British Columbia

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INTRODUCTION

Primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC) constitute a triad of liver diseases that are widely accepted to be of autoimmune etiology with differing targets of immunologic attack. In PBC, it is the microscopic intra-hepatic bile duct that is the immunologic target, whereas in PSC, both intra- and extra-hepatic bile ducts are involved and in AIH, the antigenic target is located on the hepatocyte. Aside from the fact that all three diseases will result in end-stage cirrhosis, resulting in morbidity and premature mortality unless rescued by an increasingly scarce liver transplant, there are similarities between all these three liver diseases. Overlap syndrome, in which a patient may have features of both PBC and AIH[1] or PSC and AIH[2], is well recognized to occur and is often seen by clinicians. Occasionally, patients have been reported to have overlap with PBC and PSC as well as all three[3]. Furthermore, it is well recognized that patients with PBC and AIH can have many associated extra-hepatic autoimmune disease, especially rheumatologic conditions[4,5]. PSC, although classically occurring in the context of ulcerative colitis, is also reported to be associated with an increased likelihood of co-existent extra-intestinal, extra-hepatic autoimmune diseases compared to those with ulcerative colitis alone[6]. Recently, we have reported evidence that suggests that the Aboriginal population of British Columbia (BC), Canada (i.e. part of Canada's First Nations) has a significant burden of PBC[7-9] and possibly AIH[10,11]. It is commonly accepted by the gastroenterology community of BC that inflammatory bowel disease is extremely rare amongst its First Nations peoples, a fact that most likely explains the rarity of PSC in the First Nations population.

The finding of an increased rate of PBC and AIH in BC's First Nations community has clinical, epidemiologic, anthropologic as well as socio-political implications. First Nations of Canada, also referred to as American Indians or Native Americans in the United States, are the original inhabitants of the continent and pre-date, by millennia, the subsequent European colonizers. In Canada, the First Nations peoples constitute about 4% of the population. In British Columbia, with a total population of close to 4 million people, about 170 000 are First Nations in demographic background. Often plagued by socioeconomic injustice, it is well recognized in recent years that the global health of Canada's First Nations communities is disproportionately worse than the rest of Canadian society. Chronic health problems, including diabetes mellitus, obesity, coronary artery disease, etc, are over-represented in our First Nations communities[12,13]. Excessive alcohol consumption remains a problem amongst some within the First Nations communities[14], however, the stereotypic generalization that all chronic liver diseases amongst First Nations are a result of alcoholic liver disease more likely

reflects societal bias or arises from clinical encounters in poor urban inner city areas. Within our own studies[7-9], we found that some PBC patients were initially misdiagnosed as suffering from alcoholic liver disease. The potential for accidental perpetuation of this stereotype remains in BC, since according to National Vital Statistics, all deaths from chronic liver disease are collected under the category of "alcohol-related" under a separate category of "Digestive Diseases"[15], the distinction between the two being unclear.

PRIMARY BILIARY CIRRHOSIS AND THE FIRST NATIONS COMMU-NITIES OF BRITISH COLUMBIA

Prior to the work formally studying the question of PBC amongst BC's First Nation Community, there had been indirect evidence suggesting that autoimmune liver disease was relatively common in this community. The British Columbia Vital Statistics Report[15] from 1991-1999 reported that deaths from chronic liver disease amongst First Nations women were five times higher than that in non-Aboriginal women, an interesting finding since both PBC and AIH are overwhelmingly afflictions amongst women. Furthermore, a study of the racial demographics of solid organ transplantation in British Columbia noted that organ recipients of First Nations background were significantly more likely to receive a liver allograft than any other transplanted organ (i.e. kidney, heart, lung)[16]. Lastly, anecdotal reports amongst BC's gastroenterologists[17] suggested that PBC was common amongst the province's First Nations community. The first direct evidence that PBC may be more common amongst BC's First Nations community came from the referral database of the BC Transplant Society's Liver Transplant Program[7]. It was discovered that of all patients referred for liver transplant assessment with a diagnosis of PBC, regardless of whether transplantation occurred or not, 25% were First Nation in demographic background. This was statistically significant compared to the 3.8% demographic proportion of First Nations Aboriginal's within BC's population as a whole. Not surprisingly, over half of all liver transplant recipients, who were First Nations in background, had a pre-transplant diagnosis of PBC[7]. A subsequent review of the BC Transplant Society referral database, performed a few years later, again confirmed that the trend of PBC referrals amongst the First Nations population was disproportionate to the expected[8], with a population odds ratio calculated at 8.3 with a 95% confidence interval between 5.6-12.21. The discovery, independent of the BC Transplant Society database, of an extended First Nations family with seven PBC cases in three sibships, related via common great grandparents led to a formal medical genetics population-based study of PBC in this community. This study[9] found a family history of PBC in 33% of PBC First Nations patients. A segregation analysis, which included five multiplex families, calculated the recurrence risk for PBC for all first-degree relatives, for first-degree relatives over the age of 21 years and for females over the age of 21 years as 4%, 4% and 10%, respectively. A calculation of the risk ratio (λ), an estimate of genetic effect in complex traits based on the frequency in families, compared to the population prevalence, yielded a λ for siblings of 1500, if one used a PBC population prevalence of 22 per million as previously reported in Ontario, Canada[18], and 100 if one used a PBC population prevalence of 289 per million as reported in the state of Alaska[19] which is geographically adjacent to BC. Considering that most autoimmune diseases have a λ between 8 and 20[18], a strong genetic predisposition of PBC amongst BC's First Nations population is implied.

We note that no other Canadian province has reported the finding of an increased prevalence of PBC in its First Nations community. In fact, the most well-known Canadian epidemiological study of PBC, based in the province of Ontario[19], did not report a significant association of PBC amongst First Nations. This leads to the question of whether an association of PBC in BC truly exists or whether the association is a spurious false positive one. Of importance, the First Nations communities of Canada do not constitute one homogeneous entity. From an anthropologic perspective, geographic distribution of the First Nations peoples of North American follows different temporal waves of migration from Asia. Culturally and linguistically, similarities exist within geographically linked communities and disparities exist when comparing geographically removed First Nations communities. It may not be surprising that BC's First Nations community differ in medical disease predisposition compared to Ontario's communities. Our studies have suggested that the distribution of PBC amongst BC's First Nations communities is not geographically uniform throughout the province and the predilection appears strongest amongst the communities along the Pacific Coastal areas as opposed to the non-coastal interior of BC[8,9]. We note that the American Indians of Southeast Alaska have also been reported to have an increased prevalence of PBC[20]. Southeast Alaska (i.e. the Alaskan Panhandle) is geographically adjacent to the Pacific Coast of BC where most of our First Nations patients with PBC are located. Moreover, there are cultural and anthropologic similarities between these Alaskan and BC coastal communities that appear to share a predisposition to PBC. Furthermore, it is interesting that the First Nations communities of BC' s Pacific Northwest and Southeast Alaska have both been long recognized to suffer a disproportionate burden of rheumatologic diseases[21-23] including lupus and arthritis. In general, similar rheumatologic diseases are well-known to be associated with PBC[4], suggesting that the independent reports of PBC and rheumatologic diseases within geographically proximate First Nations communities may not be spurious.

AUTOIMMUNE HEPATITIS AND THE FIRST NATIONS COMMUNITIES OF BRITISH COLUMBIA

Studies on AIH in BC's First Nations communities are still preliminary, however, there is a suggestion that this population may also be at risk for AIH. Two separate and independent database reviews have yielded similar proportions of First Nations patients amongst the BC AIH population. The first database review was of the BC Transplant Society's liver transplant candidate referrals[10] and the second was of the BC Children's Hospital AIH

Database[11]. Despite the fact that both database reviews looked at separate populations of AIH patients (i.e. adult *versus* pediatric), the proportion of First Nations with AIH was strikingly similar, approximately 17% in both. Since AIH is, in most cases, readily treated with corticosteroids and other immunosuppressive medications, unlike the situation of PBC where definitive cirrhosis-preventing treatment is not available, reviewing transplant centre and quaternary centre databases may be more prone to selection and referral bias. The discovery of classic PBC and classic AIH in a First Nations mother-daughter pair[24], although anecdotal, dramatically suggests that these autoimmune diseases are both related and associated with BC's First Nations community.

CONCLUSION

In closing, the story of PBC in BC's First Nations community underlines several important points. The first is that North American's First Nations people may have a genetic predisposition to autoimmune disease. Non-genetic factors, including recent changes in nutritional/dietary habits, environmental change/contamination, infectious agents, *etc*, may also be co-factors that interact with a genetic predisposition to produce phenotypic disease. For researchers, the reasons for disease association remain to be determined and should be the subject of further epidemiologic, basic science and clinical research. In order to accomplish this, however, researchers must be sensitive to the needs and concerns of the First Nations community. If the community is to be expected to participate, then it is a reasonable expectation that the community will, in the long-term, derive some benefit from its participation. For clinicians, a high index of suspicion is required when encountering members of an at-risk population group as is the case of chronic liver disease and BC's First Nations community. Secondly, the First Nations communities across North America are not homogeneous from a cultural anthropologic perspective and the same may also be true with regards to disease predisposition. Lastly, the dynamic interaction between medical culture and medical research can not be down-played. Currently in BC, when encountering a First Nations patient with chronic liver disease, screening for PBC and AIH is considered mandatory and alcoholic liver disease is a diagnosis of exclusion. The same may not have been true in the past.

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TOPIC HIGHLIGHT

Vito Annese, MD, *Series Editor*

Genetics of inflammatory bowel disease: The role of the HLA complex

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Abstract

The human leucocyte antigen (HLA) complex on chromosome 6p21.3 is the most extensively studied genetic region in Inflammatory bowel disease (IBD). Consistent evidence of linkage to IBD3 (6p21.1-23), an area which encompasses the HLA complex, has been demonstrated for both Crohn's disease and ulcerative colitis, and a number of replicated associations with disease susceptibility and phenotype have recently emerged. However, despite these efforts the HLA susceptibility gene (s) for IBD remain elusive, a consequence of strong linkage disequilibrium, extensive polymorphism and high gene density across this region. This article reviews current knowledge of the role of HLA complex genes in IBD susceptibility and phenotype, and discusses the factors currently limiting the translation of this knowledge to clinical practice.

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Key words: Inflammatory bowel disease; Human leucocyte antigen complex; Crohn's disease; Ulcerative colitis; Genetics

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INTRODUCTION

The human leucocyte antigen (HLA) complex is a highly polymorphic, gene dense region on chromosome 6p21.3 (Figure 1). The complex spans ~4 Mb and encompasses at least 130 expressed genes[1,2]. These include the highly polymorphic classical class Ⅰ and Ⅱ HLA genes essential for normal lymphocyte function, and a number of other genes with immunoregulatory function. Since the first report of an HLA association with IBD in 1972[3], more than 100 studies have been published investigating the role of HLA genes in determining susceptibility and phenotype of inflammatory bowel disease (IBD). Early data derived from both association and linkage analyses were inconsistent, reflecting the difficulties of inadequate sample size, low resolution typing techniques, poor statistical methodology and a failure to consider disease heterogeneity. However, since the mid-1990's, advances in genotyping and computational technology, combined with improved study design, have been successfully applied to much larger, more accurately characterised cohorts of patients, and this has revealed important clues to molecular pathogenesis of IBD.

Genome-wide scans have shown consistent evidence of linkage to IBD3 (6p21.1-23), an area which encompasses the HLA complex. This has been demonstrated in several independent studies of both Crohn's disease[4-8] and ulcerative colitis[5-8], and was recently been confirmed by the IBD Consortium in a large replication study of 733 nuclear families[9]. The importance of this area was further highlighted by a meta-analysis of 10 published genome-wide scans[10]. Notably in this study, IBD3 was the only locus that met genome-wide significance, and provided stronger evidence of linkage than 16p13.1-16q12.2 (IBD1), the locus that contains the susceptibility gene CARD15. Although it is difficult to estimate the importance of this region in determining overall genetic susceptibility, calculations derived from studies of HLA allele sharing within families suggest that this region contributes between 10%-33% of the total genetic risk of Crohn's disease[11] and 64%-100% of the total genetic risk of ulcerative colitis[12].

Interest in the HLA complex in IBD has traditionally focused on association with the classical class Ⅱ HLA alleles, but recent insights into the biological function of other genes encoded within this region have led investigators to a more diverse exploration of this region.

CLASSICAL HLA CLASS II GENES

The classical class Ⅱ HLA genes encode cell-surface

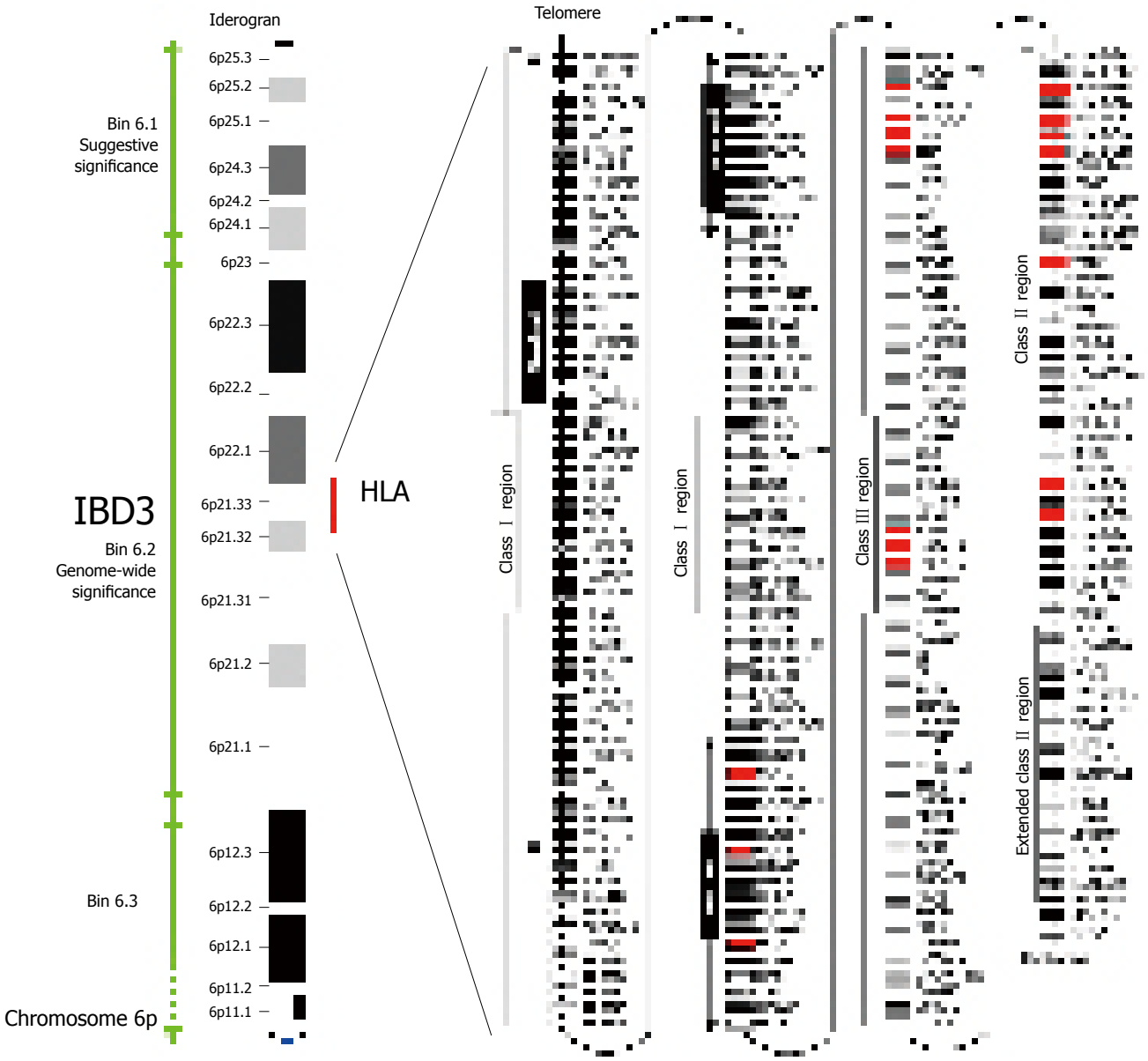


Figure 1 Crohn's disease map of chromosome 6p and the HLA region. The IBD susceptibility locus IBD3 is here defined by a 34cM chromosomal bin (6.2), which reached genome wide significance for IBD in the genome scan meta-analysis of van Heel *et al*[10]. The IBD3 region encompasses the ~ 4MB HLA region shown in detail on the right of the figure. White, grey, striped and black boxes show expressed genes, gene candidates, non-coding genes and pseudogenes, respectively. Genes with polymorphisms associate with IBD are shown in red. Modified with permission from Shiina *et al*[61].

glycoproteins which are expressed on antigen presenting cells, including dendritic cells and macrophages. Their major role is to present peptides to T cell receptors, as a prelude to T cell activation. There are three types of HLA Class Ⅱ molecules expressed by a single cell, namely HLA-DR, HLA-DQ and HLA-DP. These are each made up of αβ heterodimers: the β chains are encoded by the DRB1, DQB1 and DPB1 genes respectively, all of which are highly polymorphic. The α chains for DPA and DQA are also highly polymorphic, while the DRA gene is invariant. In addition, gene duplication events have occurred on certain haplotypes producing additional DRB3, 4 and 5 genes. When present, these are expressed at low levels in conjunction with the DRA chain.

The mechanism by which classical HLA class Ⅱ genes exert their influence in IBD is unclear, although a number of hypotheses have been postulated. Polymorphism in these molecules is concentrated around specific pockets of the binding groove that interact with critical side-chains or 'anchor' residues of a peptide. Thus different HLA molecules may bind preferentially to different peptides, or bind the same peptide with varying affinity. In IBD, cross reactivity (known as "molecular mimicry") may exist between the peptides derived from bacterial luminal flora and from self antigens present in the gut. This may lead to the generation of auto reactive T cells which contribute to disease pathogenesis through either stimulation or inhibition of the immune system. This mechanism is supported by identification of murine MHC-restricted CD4+ T cells reactive to enteric bacterial antigens that are able to induce colitis by adoptive transfer[13].

HLA-DRB1 is the most extensively studied gene in IBD. Convincing evidence of association has been described for a number of alleles, some of which confer

risk, whilst others are protective. Many of these were highlighted in a 1999 meta-analysis of 29 association studies published between 1966 and 1998[14]. Since publication of this report, more detailed genotype-phenotype analyses of this locus have been conducted in larger, accurately characterised, patient cohorts leading to the realisation that genetic variation within this region may explain some of the disease heterogeneity of IBD. The most consistently replicated associations are described below.

HLA CLASS II ASSOCIATIONS WITH CROHN'S DISEASE

*HLA-DRB1*07 and CARD15 negative ileal Crohn's disease*

The most consistently replicated association of Crohn's disease with a common HLA allele is with HLA-DRB1*07. The population frequency of this allele varies between 5%-29% in Europeans and North Americans but is less than 1% in Japanese. The 1998 meta-analysis demonstrated a weak association of HLA-DRB1*07 in unselected patients with Crohn's disease (OR 1.42, CI 1.16-1.74)[14], but three subsequent studies of well-characterised patients from the UK (n = 244)[15], Canada (n = 432)[16] and Spain (n = 210)[17] showed that this association is specifically with patients with ileal involvement (with or without colonic disease). When comparison was made in these 3 studies between patients with, and patients without ileal disease, the Odd's Ratios for ileal disease were 1.5[15], 1.9[16] and 2.6[17] respectively, which are equivalent to those observed for possession of a single CARD15 variant. Importantly the UK and Canadian studies provided evidence for genetic heterogeneity in ileal Crohn's disease, by demonstrating that the association with HLA-DRB1*07 is only present in patients who do not possess one of the three common Crohn's disease associated CARD15 variants[15,16].

Studies in other ethnic groups have not been as detailed. Few Jewish patients were included in the UK or Spanish studies, but data from the Canadian study suggested that this phenotypic association may also be present in this ethnic group, although the relatively small number included prevented this reaching statistical significance[16]. DRB1*07 appears not to be associated with Crohn's disease in the Japanese, although studies have been insufficiently powered to detect a modest effect of such a rare allele.

*HLA-DRB1*0103 and Colonic Crohn's disease*

HLA-DRB1*0103 is a rare allele with a frequency of less than 2% in European and white North American populations. It is present at a similar frequency in Jews but is absent in the Japanese. A strong association of HLA-DRB1*0103 with ulcerative colitis was identified in 1996[12], but not demonstrated in Crohn's disease until 2000[18], almost certainly a consequence of the disease heterogeneity of Crohn's disease and the low prevalence of this allele. This association has been widely replicated since, although studies in Jewish patients have been inconsistent[16,18]. Association with this allele is observed both on the DRB1*0103-DQB1*0301 and DRB1*0103-DQB1*0501 haplotypes, strongly implicating DRB1*0103 in disease pathogenesis. It is now clear that DRB1*0103 is strongly associated with colonic location, particularly isolated colonic disease[15-17,19]. The Odd's ratios for isolated colonic disease range from 5.1 to 18.5 in non-Jewish caucasoids[15-17]. Data from a recent study suggests that amongst British patients with isolated colonic Crohn's disease, DRB1*0103 is associated with the development of severe disease as defined by the requirement for infliximab or colectomy. Furthermore data from this study suggests this allele may predict time to surgery (*personal communication-Laura Hancock*). Despite the strength of these associations it should be noted that this allele was present in no more than 32% of patients with isolated colonic disease, indicating that whilst this marker has a high positive likelihood ratio, the negative likelihood ratio limits its clinical application. Association with perianal location and fistulizing behaviour, reported in two studies, probably reflects their clinical association with colonic disease, rather than an independent genetic association[15,17].

*HLA-DRB3*0301 - HLA-DRB1*1302*

The HLA-DRB3 gene is expressed in less than 50% of Europeans and white North Americans, and has not been extensively studied in IBD. However, a meta-analysis of three studies, each comprising less than 70 patients, supports a positive association of Crohn's disease with DRB3*0301 (OR 2.18, CI 1.25-3.80)[14]. This association has been replicated in a larger cohort of British patients (RR = 2.4)[15], although once again inadequate sample size has prevented investigators determining whether it is due to linkage disequilibrium with another disease associated DRB1 allele, DRB1*1302[14,15], or the HLA class Ⅰ allele Cw*0802[15]. Although stratification by disease phenotype is limited, data from this latter study, which awaits replication, suggests that this association is most marked in patients with perianal disease.

*HLA-DRB1*04 and CARD15 positive ileal disease?*

Data from the meta-analysis of seven small studies also demonstrated a non-significant increase in the common allele HLA-DRB1*04 in Crohn's disease (OR 1.62 CI 0.73-3.61)[14]. This association is particularly important in Japanese cohorts, in whom the strongest HLA associations are with DRB1*0405, *0410, and the linked DQB1*0401, 0402 alleles[20-22]. Association with DRB1*04 has not been widely observed in European and North American patients. However in the recent Canadian study, a weak association with DRB1*04 was identified in patients with ileal disease RR = 1.7 (1.1-2.5)[16]. It is interesting that this association was stronger in patients possessing one of the three common Crohn's disease associated CARD15 variants, which may indicate an epistatic interaction between this DRB1 allele and CARD15 in determining susceptibility to ileal Crohn's disease[16]. Genotype-phenotype analysis of this allele in the Japanese is awaited.

*HLA-DRB1*1501 – Crohn's disease protection*

Much confusion has surrounded associations of Crohn's disease with the serologically defined HLA allele DR2. DR2 includes the molecularly defined alleles HLA-DRB1*1501 and HLA-DRB1*1502, amongst others. The

allele frequency of DRB1*1501 varies between 6%-25% in European and white North American populations and 6%-10% in the Japanese. It is negatively associated with Crohn's disease which explains the earlier negative association reported with the serological antigen DR2 (OR 0.83, CI 0.70-0.09) highlighted in the 1999 meta-analysis[14]. This allele appears to confer protection against all subgroups of Crohn's disease, in all ethnic groups including Japanese.

HLA CLASS II ASSOCIATIONS WITH ULCERATIVE COLITIS

*HLA-DRB1*0103 and severe, extensive ulcerative colitis*

The most consistently replicated association of ulcerative colitis in European and American populations is with the rare allele HLA-DRB1*0103. Data from the Stokkers *et al*[14] meta-analysis, derived from only 3 studies, demonstrated a moderate association in unselected ulcerative colitis patients (OR 3.42 CI 1.52-7.69). Subsequent larger studies have confirmed this association in Spanish[23], North American[18], British[24]and Mexican cohorts[25]. This association is particularly strong in patients with extensive[24-27] or severe disease, as defined by the need for colectomy for failed medical therapy[24,25]. Amongst patients who require colectomy this allele may also be associated with a shorter mean time to surgery[24]. Once again, the frequency of this allele, even in the ulcerative colitis population, is too low to be clinically useful in predicting disease course.

*HLA-DRB1*1502*

HLA-DRB1*1502 is associated with ulcerative colitis in European[24], North American[18], Japanese[22,28] and Korean[29] populations. Although the background prevalence of this varies considerably, being highest in the Japanese (20%-25%) and lowest in Northern Europeans (less than 1%), the associated relative risk is similar in all populations (2-4.5). This interesting transracial concordance suggests this allele, or a nearby allele, is a true disease causing variant. This association is specifically with HLA-DRB1*1502, rather than HLA-DRB1*1501, and explains the earlier reported association with the serological antigen DR2 (OR 2.00, CI 1.52-2.63) highlighted in the 1999 meta-analysis[14]. These two alleles differ only at amino acid position 86 within pocket 1 of the peptide binding groove. At this position HLA-DRB1*1501 has a valine and DRB1*1502 (and DRB1*0103) a glycine. Limited data suggests HLA-DRB1*1502 is associated with extensive and intractable ulcerative colitis amongst Japanese[30], but not Korean patients[29].

In Japanese[31] and British UC patients[24], association has also been reported with HLA-B*52, the class Ⅰ allele found in linkage disequilibrium with HLA-DRB1*1502. However, studies have been insufficiently powered to determine which allele represents the primary association due to the highly conserved nature of this haplotype. Interestingly HLA-B*52, but not HLA-DRB1*1502, has also been shown to be associated with colonic Crohn's disease in these populations[15,32], providing further evidence of a shared genetic basis for ulcerative colitis and colonic Crohn's disease.

*HLA-DRB1*04-ulcerative colitis protection*

The common HLA-DRB1*04 allele is negatively associated with ulcerative colitis in Northern Europeans and Japanese populations, in contrast to the positive association in Crohn's disease. Data from the Stokkers meta-analysis of 15 studies demonstrated an Odd's Ratio of 0.54 (CI 0.73-3.61) in ulcerative colitis[14]. In the British population, the protective effect of this allele is confined to the most prevalent subtype DRB1*0401, and only then, when present on the two locus haplotype DRB1*0401-DQB1*0301[24]. This suggests that either an interaction between DRB1*0401 and DQB1*0301 is important for disease protection, or, and perhaps more likely, that the true protective polymorphism is found nearby on the associated extended haplotype.

HLA CLASS II ASSOCIATIONS WITH THE EXTRA-INTESTINAL MANIFESTATIONS

A number of HLA associations have been described with the extra-intestinal manifestations of IBD. The majority of data has been derived from small studies from one single centre and should be interpreted with caution until replicated. Small sample size has also hindered attempts at determining the primary association, but the most convincing associations have been described with DRB1*0103[33] in mixed studies of ulcerative colitis and Crohn's disease. Type 1 peripheral arthritis, a migratory pauciarticular large joint arthritis has specifically been shown to be associated with HLA-DRB1*0103, as well as the class Ⅰ alleles that may be found in linkage disequilibrium, namely B*27 and B*35[33]. In this study, HLA-DRB1*0103 was found in 35% of patients compared to 3% of controls. In patients with recurrent arthritis this association becomes even stronger, being found in 65% of patients[33]. In contrast, type Ⅱ peripheral arthritis, a chronic, small joint, symmetrical arthritis is associated with HLA-B*44[33]. Uveitis has also been associated with DRB1*0103 and HLA-B*27, and erythema nodosum with the TNF promoter SNP TNF-1031C[34]. However, it is important to note that an increased prevalence of extra-intestinal manifestations has previously been reported in patients with colonic Crohn's disease[35] raising the possibility that the association between DRB1*0103 and the extra-intestinal manifestations may merely reflect the replicated association with colonic disease. Further work is required to clarify this issue.

OBSERVATIONS ON HLA CLASS II ASSOCIATIONS WITH IBD

A number of important observations should be noted from the reported associations with HLA Class Ⅱ alleles described above. Firstly, the specific associations with Crohn's disease and ulcerative colitis are different, with the notable exceptions of the shared association

of ulcerative colitis and colonic Crohn's disease with HLA-DRB1*0103 and HLA-B*52. Secondly it is clear that multiple alleles, rather than a single allele, may be associated with any specific phenotype. Whilst these associations may merely reflect linkage disequilibrium with a nearby gene, this observation supports peptide presentation as an explanation for these associations, and raises the possibility of a shared disease-associated epitope, as seen in rheumatoid arthritis[36]. Some associated class Ⅱ alleles do indeed share significant residue changes in the third hypervariable region, which forms part of the antigen binding domain. However these are not shared by all of the associated alleles, suggesting this hypothesis cannot entirely explain these associations. Thirdly, it is not known whether a gene dose effect operates, such that possession of two HLA risk alleles confers additional risk. To date studies in IBD have not been sufficiently powered to conclusively answer this question. Fourthly, whilst many of the associations appear to be robust, it is clear that the penetrance of the genotype is low, and the presence of a risk allele is neither necessary nor sufficient for disease to occur. Finally the reported associations vary with ethnicity and geographical location, reflecting genetic heterogeneity, prevalence of risk alleles in the background population, and population specific patterns of linkage disequilibrium across the HLA. This illustrates the importance of studying ethnically homogenous cohorts in order to prevent population stratification.

OTHER IBD GENES IN THE HLA COMPLEX

In IBD, as with a number of other HLA Class Ⅱ -associated diseases, there is increasing evidence for more than one susceptibility locus within the HLA complex[37-41]. The evidence is accumulating faster for Crohn's disease than ulcerative colitis, reflecting the focus of recent research efforts towards Crohn's disease. Such a gene(s) might act independently of the class Ⅱ locus, or interact with disease associated class Ⅱ alleles on either the *cis* or *trans* chromosome. However, the presence of highly conserved haplotypes across such a gene-dense region has made it extremely difficult to dissect out primary associations. In Crohn's disease, it is most likely that a second disease-susceptibility locus is located between the telomeric end of class Ⅰ, and the central class Ⅲ regions. Specific associations have been identified with the class Ⅰ genes HLA-B and Cw, the non-classical MHC class Ⅰ-related (MIC) genes, MICA and MICB, and the three heat shock protein genes (HSPA1L, HSPA1A, HSPA1B). However most recent interest has focused on a cluster of immunoregulatory genes centred in the HLA class Ⅲ region, including TNF, LTA, LTB, NFKBIL and BAT1. To date most studies have investigated only a few SNPs in a single gene, chosen because of previously described associations, or for ease of genotyping, rather than their ability to 'tag' a haplotype to comprehensively survey the linkage disequilibrium across the gene. Results have generally been inconsistent, even within phenotypically defined subgroups, and as with other HLA associated diseases, very few studies have fully controlled for linkage disequilibrium with HLA-DRB1. Associations with TNF and the MIC genes are now discussed.

TNF

The most widely studied gene in the HLA class Ⅲ region is TNF, which encodes a proinflammatory cytokine that is found in increased concentrations in the mucosa, serum and stool of patients with IBD. The ΔARE[42] and $TNF^{-/-}$ mice[43] models provide experimental evidence of the importance of TNF in IBD, and convincing clinical evidence is seen in both Crohn's disease and ulcerative colitis from the dramatic response following infusion of the anti-TNFα monoclonal antibody, infliximab. A large number of TNF promoter polymorphisms have been described, raising the possibility that altered TNF expression may play a role in determining susceptibility to IBD. However the functional significance of individual polymorphisms remains uncertain as TNF regulation is highly cell and context-specific and currently available in vitro systems provide limited insight into how TNF is regulated *in vivo*.

The most consistent TNF association is with the -857C allele. This is a very common allele: 99% of the healthy caucasoid population possess at least one copy and ~85% are homozygote. A modest association with the -857C allele genotype was reported in TDT and case-control analyses from the UK. Relative risks for the homozygote TNF-857CC genotype were 2.4 (CI 1.4-4.0) in ulcerative colitis and 2.4 (CI 1.4-4.2) in Crohn's disease patients lacking the three associated CARD15 variants[44]. This association was replicated in a TDT study from Australia, but unlike the British study, evidence for epistasis was observed between TNF-857C and CARD15 variants[45]. Further evidence for an epistatic interaction was noted in a recent large TDT study from the IBD consortium, in which the TNF-857C allele was shown to be associated with Crohn's disease, but only in CARD15 transmitting families[9]. No association was found in ulcerative colitis, possibly a consequence of the smaller number of ulcerative colitis families included. At present the TNF-857CC genotype has not been found to be associated with a specific Crohn's disease or ulcerative colitis phenotype. An important confounder, which has not been directly investigated by either of these studies, is that the rare, protective -857T allele is the linkage disequilibrium with DRB1*0301[46], an allele shown by several studies to confer protection to Crohn's disease. Thus it is possible that the TNF-857C association simply reflects linkage disequilibrium within this highly complex region.

Modest associations have also been reported with many other polymorphic sites in the TNF promoter. In Crohn's disease these include TNF-1031C[47], TNF-863A[48] and TNF-308A[15,49,50]. The association with TNF-308A appears to be specifically associated with colonic Crohn's disease (with or without colonic disease)[15,49,50], however once again this finding is confounded by the tight linkage disequilibrium across the A1-B8-DR3 ancestral haplotype, the only common haplotype to contain the TNF-308A allele. In small studies TNF-308A has also been shown to be associated with ulcerative colitis[51,52] providing further evidence of shared genetic susceptibility for ulcerative colitis and colonic Crohn's disease. TNF promoter

polymorphisms may also predict ulcerative colitis phenotype: In a British study, which requires replication, homozygosity for a common promoter haplotype, defined by the common alleles at each of 6 positions, were more likely to have distal disease that remained distal throughout the period of follow-up[24].

MICA and MICB

The non-classical MHC class Ⅰ-related (MIC) genes, MICA and MICB, are expressed on the basolateral cell surface of the gastrointestinal epithelium as well as fibroblasts, endothelial cells and dendritic cells. Their expression is markedly increased in response to cellular stress including both viral and bacterial infection[53,54]. MICA and MICB bind to an activating receptor NKG2D, which is expressed on NK cells, CD8 αβ T cells, γδT cells and activated macrophages[55,56]. Interaction between these receptors may directly stimulate cell cytotoxicity as well as providing costimulation for NK and T cell activation.

Exons 2, 3 and 4 of MICA and MICB are highly polymorphic, and 54 MICA alleles have been officially designated to date. The functional significance of these polymorphisms and the nature of selective forces maintaining them are not known, but the presence of a high proportion of non-synonymous substitutions suggests that these polymorphisms are not the result of random mutation alone. Importantly, several MICA alleles have been shown to alter the binding affinity with NKG2D by as much as 30 fold, suggesting they might exert a functional effect on immune activation[57]. Studies involving unselected IBD cohorts from Europe have failed to report consistent associations with MICA or MICB polymorphisms[58-60]. However, in the Japanese, a consistent association has been reported with MICA-A6 and UC, although this does not appear to be independent of the association with the adjacent HLA-B52 allele[31]. In a phenotypically characterised cohort from the UK, MICA*010 and B*1501, which are in linkage disequilibrium, were shown to be associated with perianal Crohn's disease (RR = 2.1)[15], a finding which requires confirmation.

DOES HLA DETERMINATION HAVE A ROLE IN CLINICAL PRACTICE?

Clinical observation, supported by molecular and serological data, strongly suggests that IBD is a heterogeneous family of inflammatory disorders. Ultimately, it is hoped that more accurate clinical, serological and molecular definition of these disorders will lead to better understanding of the different biological mechanisms and complex environmental interactions specific to disease subgroups. This would assist clinicians in the prediction of disease course, prognosis, complications and response to therapy. The data reviewed in this report demonstrate that genes in the HLA are important in determining susceptibility and phenotype of Crohn's disease and ulcerative colitis. Although the specific disease causing gene(s) remain unidentified, the strength and consistency of the emerging HLA associations have confirmed the importance of classifying patients both by accurately defined clinical characteristics, and by possession of known genetic risk factors, such as CARD15 disease associated variants.

In both Crohn's disease and ulcerative colitis, the most consistently replicated associations are with the classical class Ⅱ gene, HLA-DR. In Crohn's disease the strongest associations are observed with subgroups defined by location of disease. No consistent associations have been reported with disease behaviour, a less stable phenotypic characteristic, or with age at diagnosis. At present, there is little information about HLA associations with complications or response to treatment in Crohn's disease. In ulcerative colitis, the strongest HLA associations are seen with overall susceptibility, which is consistently associated with two alleles, DRB1*0103 and DRB1*1502. These alleles vary widely in prevalence across ethnic groups. Fewer subgroup associations have been identified than with Crohn's disease, but the association with DRB1*0103 is particularly strong in patients with extensive or severe disease. However, the low sensitivity and specificity of these associations currently preclude their use as a tool in diagnosis of either Crohn's disease or ulcerative colitis. For the same reason, it is unlikely that the use of HLA markers alone will be useful in disease screening of asymptomatic, genetically high risk individuals.

In addition to the interest in using biomarkers to predict IBD susceptibility, there is also considerable interest in using molecular and serological markers to assist in the discrimination of Crohn's disease from ulcerative colitis. Currently, markers in the HLA cannot be reliably used for this purpose. Indeed the shared association of DRB1*0103 and B*52 with ulcerative colitis and colonic Crohn's disease suggests the presence of at least one shared HLA susceptibility factor, providing a tantalising clue as to the potential molecular basis for the definition of colonic inflammation. Such shared HLA factors may explain in part why both forms of IBD can coexist in a family at a frequency greater than expected by chance. The apparent differential effect of HLA-DRB1*0401, which is a susceptibility allele in Crohn's disease and a protective allele in ulcerative colitis is interesting, but weak, population specific, and neither sufficiently sensitive or specific to be clinically useful.

In the near future, knowledge of an individual's HLA genotype is likely to be most useful in the prediction of disease course in patients with an established diagnosis of Crohn's disease or ulcerative colitis. However, at present, the sensitivity and specificity of even the most robust associations limit their application in isolation in this clinical context. Integration of an individual's HLA genotype into a panel of other genetic and serological markers may improve the sensitivity and specificity, although ultimately this is likely to require the identification of the primary IBD genes within this complex region.

In common with virtually all other HLA associated complex diseases, the HLA susceptibility gene(s) for IBD remain elusive. This is a consequence of the obstacles that challenge the mapping of disease genes within this region, including the high gene density and degree of polymorphism; the complex haplotype structure and

patterns of linkage disequilibrium; the relatively small relative risks conferred by disease associated alleles; and the likely clustering of more than one IBD susceptibility gene, either within the HLA, or within the surrounding IBD3 locus.

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TOPIC HIGHLIGHT

Vito Annese, MD, *Series Editor*

Multidrug resistance 1 gene in inflammatory bowel disease: A meta-analysis

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Abstract

The MDR1 gene is an attractive candidate gene for the pathogenesis of inflammatory bowel disease (IBD) and perhaps response to therapy, with evidences at both functional and genetic levels. Its product, the P-glycoprotein (P-gp) functions as a transmembrane efflux pump thus influencing disposition and response of many drugs, some of whom (i.e. glucocorticoids) central to IBD therapy. In addition P-gp is highly expressed in many epithelial surfaces, included gastrointestinal tract (G-I) with a putative role in decreasing the absorption of endogenous or exogenous toxins, and perhaps host-bacteria interaction. Many genetic variations of MDR1 gene has been described and in some instances evidences for different P-gp expression as well drugs metabolism have been provided. However data are often conflicting due to genetic heterogeneity and different methodologies employed. Perhaps the greatest piece of evidence of the physiological importance of P-gp in the G-I tract has come from the description of the mdr1 knock-out mice model, which develops a spontaneous colitis in a specific pathogen-free environment. Studies investigating MDR1 gene polymorphism and predisposition to IBD have also shown conflicting results, owing to the known difficulties in complex diseases, especially when the supposed genetic contribution is weak. In this study we have undertaken a meta-analysis of the available findings obtained with two SNPs polymorphism (C3435T and G2677T/A) in IBD; a significant association of 3435T allele and 3435TT genotype has been found with UC (OR = 1.17, P = 0.003 and OR = 1.36, P = 0.017, respectively). In contrast no association with CD and the G2677T/A polymorphism could be demonstrated.

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INTRODUCTION

The role of P-glycoprotein (P-gp), the encoded product of the MDR1 gene, has been extensively studied as a mediator of multidrug resistance phenotype associated with certain types of cancers. Constitutive expression of this transporter in normal tissues has been shown to play an important role in drug disposition and response. More recently, genetic heterogeneity in terms of single nucleotide polymorphisms (SNPs) in this transporter has received significant attention as potential determinant of susceptibility to inflammatory bowel disease (IBD), and variability in drug efficacy in this condition.

The goal of this review is to summarize the available knowledge regarding the role of P-gp in the human gastrointestinal tract and on drug disposition process, and to focus specifically on the putative effects of the MDR1 gene polymorphisms in IBD by means of a meta-analysis of the available studies.

P-GP FUNCTION

P-gp is a member of the adenosine triphosphate (ATP)-binding cassette family that is encoded by the human ABCB1 gene (ATP-binding cassette, subfamily B), also called MDR1[1]. Human P-gp is a phosphorylated and glycosilated transmembrane protein consisting of 1280 amino acids and 2 homologous and symmetric sequences, each containing 6 transmembrane domains and an ATP-binding motif. P-gp functions as a transmembrane efflux pump, thereby moving drugs from the intracellular to the extra cellular domain; it may also interact with drug molecules trapped within the cell membrane lipid bilayer. ATP hydrolysis provides the energy for active drug transport against steep concentration gradient

(like a "vacuum cleaner")[2]. P-gp was first isolated from colchicine-resistant Chinese hamster ovary cells[3]. Subsequently the gene coding for P-gp (MDR1) was identified owing to its over expression in tumour cells associated with an acquired cross-resistance to multiple cytotoxic anticancer agents[4]. In humans, two MDR genes, MDR1 and MDR3 (also called MDR2), have been described, whereas in rodents three genes mdr1a, mdr1b, mdr2, have been identified. Subsequently, P-gp was also recognized to be expressed in many normal tissues, such as the canalicular surface of hepatocytes, the apical surface of proximal tubular cells in kidneys, and the brush borders of the enterocytes[5]. In addition, P-gp is also found in the epithelium of the brain choroids plexus (where constitutes the blood-cerebrospinal fluid barrier) as well as on the luminal surface of blood capillaries of the brain (blood-brain barrier)[6]. P-gp is also expressed in other tissues known to have blood-tissues barriers, such as placenta, ovaries, and testes[7]. Moreover, P-gp has been detected in haematopoietic stem cells, peripheral blood mononuclear cells, mature macrophage, natural killer cells, antigen-presenting dendritic cells, and T and B-lymphocytes[8].

The function and especially the anatomic localization of P-gp suggest that this transporter acts as a protective barrier to keep toxins out of the body by secreting them into bile, urine, and intestinal lumen, and thereby preventing their accumulation in critical organs[9].

P-gp IN THE GASTROINTESTINAL TRACT

In the human gastrointestinal tract (GI), P-gp is found at high concentrations on apical surfaces of superficial columnar epithelial cells of the colon and distal small bowel. High levels are also found in small biliary ductules and small pancreatic ductules[5]. In the GI tract there is a regional variation in P-gp expression with a maximal expression in the epithelial cells of the ileum and a gradual decline proximally to the jejunum, duodenum, and stomach; variations across the colon are less well defined[10]. The putative role of the P-gp in the GI tract is to decrease the absorption of endogenous and exogenous hydrophobic amphipathic toxins[11,12]. A great insight into the physiological role of P-gp protein in the GI tract has derived from the phenotype of the mdr1a gene knockout mice. This model was prompted by the finding that the MDR1 gene is present in a region of the human genome (7q21.1) that may harbour a disease gene involved in susceptibility to IBD[13]. Interestingly, the mdr1a knockout mice also develop a spontaneous colitis when maintained under specific pathogen-free conditions[14]. The colitis is prevented and reversed by the administration of antibiotics, suggesting that the intestinal flora is necessary to initiate and perpetuate the inflammation. This model implies that loss of the xenobiotic efflux mechanism may promote the development of the colitis. At histology, presence of long and dysregulated crypts, crypt abscess, and superficial mucosal inflammation has been described, a picture reminiscent of ulcerative colitis. However, transmural infiltration resembling Crohn's disease has also been observed. More recently it has been described that Helicobacter bilis infection accelerated while Helicobacter hepaticus infection delayed the development of spontaneous colitis[15], thus suggesting that specific luminal bacteria may critically influence the development of colitis in genetically susceptible animals.

Table 1 Substrates (S), inhibitors (I-) and inducers (I+) of P-glycoprotein (Modified from ref. 9)

DRUG	S	I-	I+	DRUG	S	I-	I+
Anticancer				**Antiviral**			
Actinomycin D	√			Amprenavir	√		√
Daunorubicin	√			Indinavir	√	√	√
Mitomicyn C	√			Nelfinavir	√	√	√
Mitoxantrone	√			**Antibiotics**			
Vinblastine	√			Claritromycin		√	
Vincristine	√			Erythromycin	√	√	
Antihypertensive				Levofloxacin	√		
Diltiazem	√			Rifampin	√		√
Losartan	√			Tetracycline	√		
Nicardipine		√		**Antimycotics**			
Talinolol	±			Itraconazole	√	√	
Antiarrhythmics				Ketoconazole		√	
Amiodarone		√		**Immunosup**.			
Digoxin	√			Cyclosporine	√	√	
Quinidine	√	√		Tacrolimus	√	√	
Verapamil	√	√		Methotrexate	√		
Glucocorticoids				**Antidepressants**			
Aldosterone	√			Amitriptyline	√		
Cortisol	√			Fluoxetine		√	
Dexamethasone	√		√	**Neuroleptics**			
Methylprednisolone	√			Chlorpromazine		√	
Others				Phenotiazine		√	
Colchicine	√			Phenobarbital	√		
Dipyridamole		√		**Opioids**			
Loperamide	√			Methadone		√	
Progesterone		√		Morphine	√		
Spironolactone		√		**Antacids**			
Domperidone	√			Ranitidine	√		
Ondansetron	√			Cimetidine	√		

P-gp SUBSTRATES

The structure-activity relationship for P-gp substrates has yet to be clearly defined. Table 1 summarizes a comprehensive list of compounds that act as substrates, inhibitors or inducers of P-gp (modified from Marzolini *et al*)[9]. The range of substrates is broad and it also evident that many drugs are also substrates of the cytochrome P450 (CYP) 3A4. The overlap between CYP3A4 and P-gp is also emphasized by the genomic proximity of both genes (7q22.1 and 7q21.1 for CYP3A4 and MDR1, respectively)[16]. The co-localization of the P-gp and CYP3A4 in the small intestine and liver suggests that this transporter also plays a significant role in the oral bioavailability, distribution, and excretion of drugs. Evidence supporting such a role was obtained from animal models. A mouse strain naturally deficient in mdr1a demonstrated a marked sensitivity to the neurotoxic effects of ivermectin, an antiparasiticide[17]. Experiments with mdr1a knockout mice have revealed the P-gp limits the central nervous system entry of drugs like cyclosporine, digoxin, vinblastine[18], and the oral absorption of drug like paclitaxel[19]. Of broad clinical relevance is the interaction

between digoxin and other cardiac drugs such as verapamil, quinidine, and amiodarone[20]. Of interest, several drugs central to IBD therapy are also MDR1 substrates like glucocorticoids[21,22], cyclosporine[23] and methotrexate[24].

POLYMORPHISM IN THE HUMAN MDR1 GENE

MDR1 is located in the human chromosome 7 band p21-21.1 on a 600 kb NruI fragment[25], and the MDR1 coding region is contained on a 120 kb XhoI fragment[26]. The gene extends over more than 100 kb containing 28 introns, 26 of them interrupting the protein-coding sequence. MDR1 mRNA has a size of 4.7 kDa, thus its coding region accounts for less than 5% of the total. The first report of MDR1 polymorphism was presented in 1989[27]. To date, genetic variations of the human MDR1 gene has been extensively studied (reviewed in ref.28, 29) with 50 SNPs and 3 insertion/deletion polymorphisms reported. Moreover, several preclinical and clinical studies have provided evidence for naturally occurring polymorphisms of MDR1 gene and their effects on drug absorption, distribution, and elimination[30]. Hoffmeyer *et al* performed the first systematic screening for MDR1 polymorphisms in 2000[31]; in that study a synonymous SNP in exon 26 (C3435T) was the first variation to be associated with altered protein expression, although the SNP does not change the encoded amino acid (Ile). In addition, P-gp expression in the duodenum of individuals with the homozygous T allele (variant) was decreased when compared with individuals with the C allele (wild type). Other studies have shown that SNPs at exon 21 (G2677T/A) and at exon 1b (T129C) may also be associated with altered transport function or expressions[32,33]. However, a disequilibrium exists between SNPs in exon 26 (C3435T) and exon 21 (G2677T/A), suggesting that the observed differences in P-gp, initially attributed to the exon 26 SNP, may be the result of the associated polymorphism in exon 21[32]. Furthermore, several groups have subsequently confirmed the existence of such distinct haplotypes[34,35], with three common haplotypes found in more than 70% of individuals. It has also been recently shown that a synonymous SNP in exon 12 (C1236T) is linked to the C3435T and G2677T/A SNPs[36,37]. Available data indicate that the allele frequencies of these three main variants differ considerably in the various populations. Of note, marked differences of the C3435T SNP allele frequency have been observed with an increased frequency of the C allele (wild type) in African populations compared with Caucasian-Asian populations[38,39]. Similarly, the 2677A genotype of the G2677T/A SNP is significantly more common in Japanese subjects[40], while the variant 1236T of the C1236T SNP is significantly more frequent in Asian subjects compared to Caucasian subjects[41].

The study of Hoffmeyer *et al*[31] was the first to demonstrate a 2-fold reduction in P-gp expression in duodenal biopsy samples among healthy Caucasian subjects homozygous for the exon 26 3435T allele. Those subjects were also shown to have increased digoxin plasma concentrations after oral administration, suggesting greater drug absorption in individuals with low intestinal P-gp levels. However, the replication of this finding has been controversial with consistent[42,43] and conflicting studies[44-46]. In a study quantifying MDR1 mRNA in the duodenum, Nakamura *et al*[47] showed, in contrast to the Hoffmeyer's findings, greater levels in healthy Japanese subjects carrying the 3435T allele as compared with subjects with the C3435T allele of the exon 26. This controversy, however, is not limited to the Asian population[34], as conflicting data have been noted also for others P-gp substrates including fexofenadine[32,48] cyclosporine[49-53] and tacrolimus[36,54-57]. Similarly conflicting data have also been reported for the exon 21 polymorphisms concerning the digoxin[44,45], tacrolimus[35,54,55-57], and multiple drugs[58,59] pharmacokinetics. Furthermore, conflicting data have also been obtained when evaluating the P-gp or mRNA mucosal expression with studies demonstrating reduced[31,60,48,61], increased[47] or unchanged[33,34,36,58,62-65] P-gp function for mutant C3435T SNP. Functional studies investigating the contribution of G2677T/A polymorphism also accounted for conflicting results[32-34,36,40,58,59,63-65]. Possible reasons for such discrepancies are the existence of gene-gene interaction, linkage disequilibrium between C3435T SNP and other MDR1 SNPs, and environmental factors influencing also the CYP enzyme activity. Moreover, it should be noted that regulation of P-gp expression might significantly differ from tissue to tissue. For example, the ligand-activated nuclear receptor PXR is though to be critical to P-gp expression in the liver and intestine, whereas in sites such as blood-brain barrier, PXR is unlikely to display regulatory functions. Finally, the methodology used to measure P-gp expression widely differs between studies. In addition different substrate drugs have been used in various studies, with different route of administration and potential different extent of metabolism relative to P-gp-mediated transport. For example, cyclosporine not only is transported by P-gp but also it is a substrate of CYP3A4, thus a potential P-GP effect may be masked by CYP3A4 activity.

MDR1 AND INFLAMMATORY BOWEL DISEASE

The MDR1 gene is an attractive candidate gene for the pathogenesis of IBD and perhaps response to therapy, with evidences at both functional and genetic levels[66]. In a German case-control study Schwab *et al* investigating the C3435T polymorphism firstly reported an increase of the T allele and TT genotype frequencies in 149 patients with ulcerative colitis, but not Crohn's disease, compared with controls ($P = 0.049$, OR = 1.4; $P = 0.005$, OR = 2.1, respectively)[67]. Subsequent studies, however, have gained conflicting results: Glas *et al*[68] in a small group of 123 patients with UC found results in partial accordance with a trend towards an increased frequency of T allele compared to healthy controls, but a statistical difference was obtained only in one of two different control groups (T allele, $P = 0.018$; TT genotype, $P = 0.016$), thus suggesting the key factor of patients and controls recruitment. In a small

Greek study[69] no difference was found in both UC and CD patients, but this study has been criticized because the investigated populations were not in Hardy-Weinberg equilibrium. A large study[70] performed in a German and British cohort investigating 307 UC and 564 CD patients by using both a case-control strategy and a transmission disequilibrium test based analysis (which is more resilient to latent effects such as population stratification), failed to demonstrate an association, even after stratifying individuals on the basis of CARD15 gene variants. More recently Ho *et al*[71] confirmed the association in UC patients ($P = 0.04$, OR 1.6 for TT genotype), especially those with extensive colitis ($P = 0.003$, OR = 2.64), but in contrast completely negative findings have been reported in large studies from North America[72], Slovenia[73] and Italy[74]. In particular in the latter study from our Institution, about one thousand IBD patients have been genotyped (Tables 2 and 3) with negative results. In addition, Urcelay *et al*[75] paradoxically found a significant association of the CC3435 genotype (wild) in CD patients ($P = 0.007$), but data were not in Hardy-Weinberg equilibrium.

In a multicenter North American cohort with 444 IBD trios, Brant *et al*[72] investigated also another polymorphism of MDR1, the tri-allelic G2677T/A SNP (Ala893Ser/Thr). They found a significant association of the Ala893 variant (G2677), known to decrease transporter function, with IBD in both case-control analysis ($P = 0.002$) and the pedigree disequilibrium test (PDT) ($P = 0.0002$), especially in non-Jewish subjects. The association was confirmed by PDT analysis within the CD subset ($P = 0.001$) with a similar, non-significant trend in the small subset of UC patients. Curiously, an association with another allele (T allele, 893Ser variant) of this SNP was found in UC patients ($P = 0.029$) by Potočnik *et al*[73]. Ho *et al*[71], did not find an association between this polymorphism and IBD; however, a 2-locus haplotype (3435T/G2677) was significantly associated with UC in this study ($P = 0.03$). Urcelay *et al*[75] similarly did not found a correlation between this polymorphism and IBD, however, a trend towards an increased frequency of 2-locus 2677T/C3435 haplotype was found in CD patients. In contrast, more recently Palmieri *et al*[74] did not found any association of this polymorphism and 2-locus haplotype with IBD patients (Table 3). Reasons for this discrepancy may lie in population heterogeneity, sample size, selection of control population, incomplete phenotype description, and difficulty to replicate results in case control study when the contribution of the genetic predisposing factor under investigation is weak. Accordingly, when looking for example at the existing data on C3435T polymorphism, it is apparent that the 5%-8% difference of the "risk" allele and genotype frequencies in UC patients and their significance (P values ranging from 0.02 to 0.049) are rather modest. More importantly, there is a significant heterogeneity in patients (T allele frequency ranging from 42.3% up to 65.3% in UC) and especially control populations (T allele frequency ranging from 35.7% up to 63%).

The hypothesis that altered PGP expression in IBD patients could modify the response to medical therapy was put forward by Farrell *et al*[76]. Peripheral blood lymphocytes (PBL) from patients with active Crohn's disease (CD) and Ulcerative colitis (UC) in whom medical therapy had failed and surgical intervention had been necessary were shown to have higher expression of P-gp glycoprotein compared with those from patients who had inactive disease and required surgery for obstruction or dysplasia. Based on this observation the authors speculated that poor response to glucocorticoids in IBD might relate in part to constitutive MDR1 expression. We have recently challenged this hypothesis in a large cohort of IBD patients[74] using steroids, by evaluating both C3435T and G2677T/A polymorphisms. Allele and genotype frequencies were compared in 594 patients using systemic steroids, subgrouped in 320 responders, 76 non-responders and 198 steroid-dependent. No significant differences were found within subgroups and between subgroups and 450 healthy subjects. Moreover, 297 patients taking immunosuppressive drugs (included azathioprine, 6-mercaptopurine, methotrexate, and cyclosporine) were also evaluated. No influence of MDR1 genotypes was found with respect to response to therapy. Similarly, in a recent study by Mc

Table 2 Distribution of C3435T alleles and genotypes (cases and controls) in the existing literature

	C	T	CC	CT	TT
Glas, 2003					
UC = 123[1]	111 (45)	135 (55)	19 (15)	73 (60)	31 (25)
CD = 135	130 (48)	140 (52)	26 (19)	78 (58)	31 (23)
IBD = 258	241 (47)	275 (53)	45 (17)	151 (59)	62 (24)
HC = 265	272 (51)	258 (49)	70 (26)	132 (50)	63 (24)
Schwab, 2003	C	T	CC	CT	TT
UC[a] = 149	129 (43)	169 (57)	26 (17)	77 (52)	46 (31)
HC = 149	154 (52)	144 (48)	39 (26)	76 (51)	34 (23)
CD = 126	134 (53)	118 (47)	33 (26)	68 (54)	25 (20)
HC = 126	128 (51)	124 (49)	35 (28)	58 (46)	33 (26)
IBD = 275	263 (48)	287 (52)	59 (21)	145 (53)	71 (26)
HC = 275	282 (51)	268 (49)	74 (27)	134 (49)	67 (24)
Ho, 2005	C	T	CC	CT	TT
UC[c] = 335	280 (42)	390 (58)	61 (18)	158 (47)	116 (35)
CD = 268	252 (47)	284 (53)	56 (21)	140 (52)	72 (27)
IBD = 603	532 (44)	674 (56)	117 (19)	298 (50)	188 (31)
HC = 370	354 (48)	386 (52)	82 (22)	190 (51)	98 (27)
Palmieri, 2005	C	T	CC	CT	TT
UC = 468	488 (52)	448 (48)	124 (27)	240 (51)	104 (22)
CD = 478	503 (53)	453 (47)	125 (26)	253 (53)	100 (21)
IBD = 946	991 (52)	901 (48)	249 (26)	493 (52)	204 (22)
HC = 450	470 (52)	430 (48)	115 (26)	240 (53)	95 (21)
Potocnik, 2004	C	T	CC	CT	TT
UC = 144	134 (47)	154 (53)	-	-	-
CD = 163	161 (49)	165 (51)	-	-	-
IBD = 307	295 (48)	319 (52)			
HC = 355	376 (53)	334 (47)	-	-	-
Urcelay, 2006					
UC = 311	317 (51)	305 (49)	87 (28)	143 (46)	81 (26)
CD[b] = 303[1]	369 (61)	237 (39)	122 (40)	125 (41)	56 (19)
IBD = 614	686 (56)	542 (44)	209 (34)	268 (44)	137 (22)
HC = 324	344 (53)	304 (47)	97 (30)	150 (46)	77 (24)

UC = Ulcerative colitis, CD = Crohn's disease, HC = healthy controls. [a]$P = 0.045$ (T *vs* C); $P = 0.049$ (TT *vs* CC). [c]$P = 0.02$ (T *vs* C); $P = 0.04$ (TT *vs* CC). [b]$P = 0.006$ (T *vs* C); $P = 0.01$ (TT *vs* CC). [1] Subjects not in Hardy-Weinberg equilibrium.

Table 3 Distribution of G2677T/A alleles and genotypes (cases and controls) in the existing literature

	A	G	T	AA	GG	GT	TT	GA	TA
Brant, 2003									
IBD_NJ[a] = 211	2 (0.5)	254 (60.2)	166 (39.3)	-	76 (36)	101 (48)	32 (15)	1 (0.5)	1 (0.5)
HC_NJ = 392	20 (2.6)	411 (52.4)	353 (45)	-	108 (27)	183 (47)	81 (21)	12 (3)	8 (2)
IBD_J = 114	1 (0.5)	143 (62.7)	84 (36.8)	-	41 (36)	60 (53)	12 (10)	1 (1)	0
HC_J = 219	13 (3)	265 (60.5)	160 (36.5)	-	85 (39)	89 (41)	32 (15)	6 (2)	7 (3)
Ho, 2005	A	G	T	AA	GG	GT	TT	GA	TA
UC = 335	-	366 (54.6)	304 (45.4)	-	95 (28.3)	176 (52.5)	64 (19.1)	-	-
CD = 268	-	283 (52.8)	253 (47.2)	-	75 (27.9)	133 (47.8)	60 (22.4)	-	-
IBD = 603	-	649 (53.8)	557 (46.2)	-	170 (28.2)	309 (51.2)	124 (20.6)	-	-
HC = 370	-	378 (51.2)	362 (49.8)	-	102 (27.6)	174 (47.0)	94 (25.4)	-	-
Palmieri, 2005	A	G	T	AA	GG	GT	TT	GA	TA
UC = 468	23 (2.5)	494 (52.8)	419 (44.7)	1 (0.2)	127 (27.1)	229 (49.0)	90 (19.2)	11 (2.4)	10 (2.1)
CD = 478	15 (1.6)	534 (55.9)	407 (42.5)	0 (0.0)	143 (29.9)	237 (49.6)	83 (17.4)	11 (2.3)	4 (0.8)
IBD = 946	38 (2)	1028 (54)	826 (44)	1 (0.1)	270 (29.5)	466 (49.2)	173 (18.3)	22 (2.3)	14 (1.5)
HC = 450	19 (2.1)	500 (55.6)	381 (42.3)	0 (0.0)	126 (28.0)	236 (52.4)	69 (15.3)	12 (2.7)	7 (1.6)
Potocnik, 2004		A & G	T						
UC = 144	-	157 (54)	131 (46)	-	-	-	-	-	-
CD = 163	-	187 (57)	139 (43)	-	-	-	-	-	-
IBD = 307	-	344 (56)	270 (44)	-	-	-	-	-	-
HC = 355	-	424 (59.7)	286 (40.3)	-	-	-	-	-	-
Urcelay, 2006									
UC = 311	4 (0.7)	372 (62.8)	216 (36.5)	0 (0)	118 (39.9)	133 (44.9)	41 (13.9)	3 (1)	1 (0.3)
CD = 303	6 (0.9)	403 (63.2)	229 (35.9)	0 (0)	139 (43.6)	122 (38.3)	52 (16.3)	3 (0.9)	3 (0.9)
IBD = 615	10 (0.8)	775 (63)	445 (36.2)	0 (0)	257 (41.8)	255 (41.5)	93 (16.1)	6 (0.9)	4 (0.7)
HC = 352	8 (1.1)	426 (60.5)	270 (38.4)	0 (0)	140 (39.8)	142 (40.3)	62 (17.6)	4 (1.1)	4 (1.1)

[a]$P = 0.03$ (G *vs* T); $P = 0.08$ (GG *vs* GT TT).

Table 4 Summary of ORs and 95% CIs for different outcomes obtained at the meta-analysis

		Fixed effects		Random effects		Heterogeneity test
		P value	OR (95% CI)	*P* value	OR (95% CI)	
	Outcome CD					0.09
C3435T	T *vs* C (6 studies)	0.519	0.968 (0.878-1.068)	0.722	0.974 (0.842-1.126)	
	TT *vs* CC (5 studies)	0.297	0.892 (0.720-1.106)	0.472	0.900 (0.675-1.200)	NS
G2677T/A	G *vs* T (4 studies)	0.635	1.027 (0.920-1.145)	0.635	1.027 (0.920-1.145)	NS
	GG *vs* GT TT (3 studies)	0.338	1.092 (0.912-1.308)	0.338	1.092 (0.912-1.308)	NS
	Outcome UC					
C3435T	T *vs* C (6 studies)	0.002	1.170 (1.062-1.289)	0.003	1.178 (1.058-1.311)	NS
	TT *vs* CC (5 studies)	0.008	1.332 (1.080-1.644)	0.017	1.367 (1.057-1.768)	NS
G2677T/A	G *vs* T (4 studies)	0.843	0.989 (0.887-1.103)	0.862	0.986 (0.836-1.162)	NS
	GG *vs* GT TT (3 studies)	0.947	0.994 (0.830-1.190)	0.947	0.994 (0.830-1.190)	NS
	Outcome IBD					
C3435T	T *vs* C (6 studies)	0.083	1.074 (0.991-1.165)	0.135	1.083 (0.976-1.201)	NS
	TT *vs* CC (5 studies)	0.225	1.116 (0.935-1.332)	0.274	1.135 (0.904-1.426)	NS
G2677T/A	G *vs* T (5 studies)	0.351	1.041 (0.957-1.132)	0.448	1.047 (0.930-1.178)	NS
	GG *vs* GT TT (4 studies)	0.366	1.065 (0.929-1.221)	0.366	1.065 (0.929-1.221)	NS

Govern *et al* in which the need for colectomy in UC patients was used as a surrogate marker for glucocorticoids resistance, no association was found with C3435T polymorphism[77].

Although the Farrell's hypothesis is attractive, data from our study strongly question the influence of MDR1 gene on response to steroid therapy in IBD patients. There are, indeed, also some caveats to the Farrell's study; firstly, circulating lymphocytes consist of different subsets of lymphocytes which express MDR1 at varying levels, since it is now established that lymphocytes homing to the gut associated lymphoid tissue are phenotypically distinct from circulatory ones. Accordingly, Yacyshyn *et al*[78] have demonstrated different level of MDR1 expression and activity in intraepithelial, lamina propria, and PBL. Overall, intraepithelial MDR1 expression and function were lower in UC compared with CD and healthy controls. Accordingly, Langman *et al* found a down-regulation of MDR1 tissue expression in UC patients[79]. Moreover, the effect of corticosteroids or other treatment used in IBD on MDR1 expression it is not fully established. It remains therefore to be evaluated whether different expression of P-gp reflects

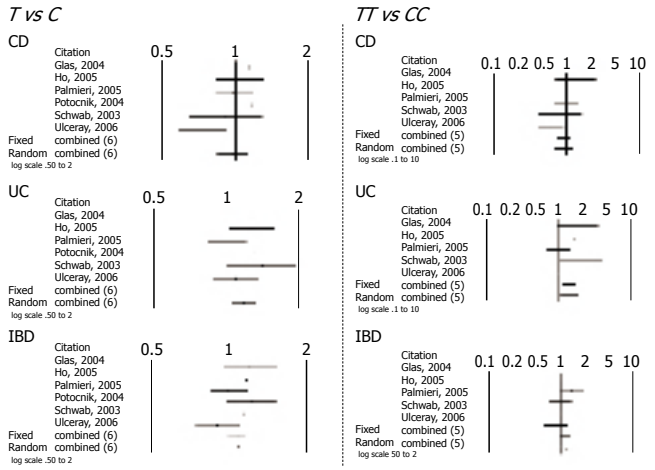


Figure 1 Schematic representation of studies included in the meta-analysis of C3435T SNP.

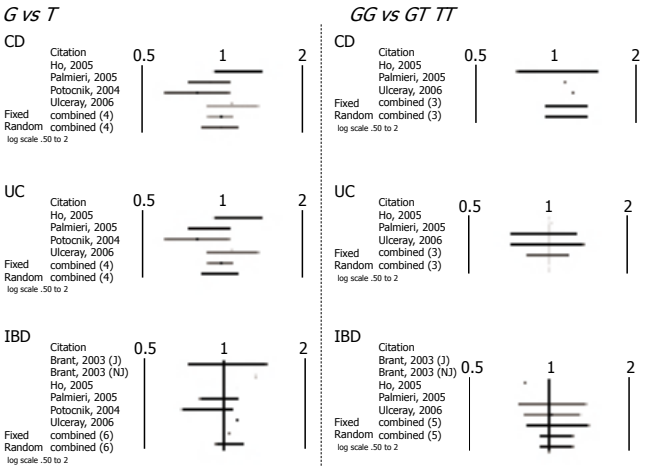


Figure 2 Schematic representation of studies included in the meta-analysis of G2677T/A SNP.

a secondary phenomenon to therapy or a primary one, influencing the response to treatment. Finally, increased expression does not necessarily imply increased function in diseased states. Based on the available evidence, the contribution of MDR1 gene on IBD predisposition and response to medical therapy, although biologically plausible is still unproven.

META-ANALYSIS

In the attempt to evaluate the potential association of C3435T and G2677T/A polymorphisms with IBD, a meta-analysis has been performed. Because case-control studies were included, the odds ratios (ORs) were employed. For each outcome, the between-study heterogeneity across all the eligible comparisons has been performed by using the chi-square based Q statistic; heterogeneity was considered significant for p lower than 0.10. Data were combined by using both fixed and random effects models; random effects are more appropriate when heterogeneity is present. Analysis was conducted by Comprehensive Meta Analysis v.1.0.23, 1999 Biostat software (www.meta-analysis.com).

In Tables 2 and 3 are depicted the characteristics of the studies included in the meta-analysis, with the allele and genotype frequencies. The study by Gazouli *et al*[69] has been excluded due to failure of Hardy-Weinberg equilibrium. The study of Croucher *et al*[70] was also excluded due to lack of data concerning allele and genotype frequencies, also upon specific request to the authors. For the study of Brant *et al*[72] data are expressed as whole IBD, since no complete information for UC and CD subgroups were available.

When comparing allele (T *vs* C) and genotype frequencies (TT *vs* CC) of the C3435T SNP in CD and UC patients, a slight but significant difference in the pooled data was found in UC patients (Table 4). More specifically a significant association with T allele (OR 1.17, CI 1.06-1.31, $P = 0.003$) and TT genotype (OR=1.36, CI 1.05-1.76, $P = 0.017$) was demonstrated. In contrast no association in CD patients was found and in IBD patients as whole. Similarly no significant difference was found after pooling data of the available studies for allele and genotype frequencies of the G2677T/A SNP (Figures 1 and 2).

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TOPIC HIGHLIGHT

Vito Annese, MD, *Series Editor*

Immunogenetic phenotypes in inflammatory bowel disease

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Abstract

The currently accepted etiopathogenic hypothesis suggests that the chronic intestinal inflammation and related systemic manifestations characteristic of inflammatory bowel disease (IBD) are due to an overly aggressive or pathologic immune response to resident luminal bacterial constituents. Predisposing factors are genetic dysregulation of mucosal immune responses and/or barrier function, with onset triggered by environmental stimuli. These factors and their interactions may also be important determinants of disease phenotype and disease progression. The emergence of immunogenetic phenotypes lends support to the proposed hypothesis that susceptibility genes regulate distinct immune processes, driven by luminal antigens, expressed as specific immune phenotypes which in turn influence clinical phenotypes in IBD patients.

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Key words: Inflammatory bowel disease; Immune reactivity; Disease phenotype

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INTRODUCTION

The currently accepted etiopathogenic hypothesis suggests that the chronic intestinal inflammation and related systemic manifestations characteristic of inflammatory bowel disease (IBD) are due to an overly aggressive or pathologic immune response to resident luminal bacterial constituents. Predisposing factors are genetic dysregulation of mucosal immune responses and/or barrier function, with onset triggered by environmental stimuli. These factors and their interactions may also be important determinants of disease phenotype and disease progression. The interaction between genetic susceptibility and luminal bacteria deserves particular attention. The results from animal studies indicate that not all commensal bacterial species are identical in their abilities to induce disease in the face of a common genetic defect. For instance, HLA B27/β2 microglobulin transgenic rats raised under sterile conditions do not develop colitis. However, when colonized with normal specific pathogen-free commensal cecal bacteria, they develop aggressive disease involving the colon and gastro-duodenal area within one month[1]. On the one hand, when colonized with a single bacterial strain, *Bacteroides vulgatus*, these same genetically susceptible rats develop moderate colitis but no gastro-duodenal disease. When selectively colonized with Eschericia.coli, they exhibit neither disease nor T cell activation[2]. This data suggests that not all commensal bacterial strains trigger an abnormal immune response for a given genetic susceptibility. The same non-pathogenic bacteria, however, may induce inflammation in a different genetically susceptible host. More specifically, further research from the same group demonstrated that Escherichia coli induced only mild cecal inflammation after 3 wk of monoassociation in interleukin 10-/- mice. In contrast, Enterococcus faecalis-monoassociated interleukin 10-/- mice developed distal colitis at 10-12 wk that was progressively more severe and associated with duodenal inflammation and obstruction by 30 wk. Their results suggest that different commensal bacterial species selectively initiate immune-mediated intestinal inflammation with distinctly different kinetics and anatomic distribution in the same host[3]. These findings highlight the fact that both genetic susceptibility and luminal antigenic drive are important determinants of disease susceptibility and modification. Immune reactivity as measured by the serological expression of immune responses to specific bacteria may indeed be representative of this host gene luminal bacterial interaction. If indeed these immune responses represent the sum of a genetic and environmental predisposition to IBD, quantitative and qualitative expression of these immune responses may serve as an immunologic risk marker for IBD phenotypes.

IMMUNE PHENOTYPES

Immune responses to resident intestinal flora in humans have been reported. Duchmann *et al* demonstrated that CD patients boast reactivity to hundreds of bacterial antigens created from sonification of multiple bacterial specifies including enterobacteria, bacteriodes and bifidobacterium[4]. Immune reactivity to more specific microbial antigens has been reported in the sera of patients with IBD (Table 1).

Antibodies to *Saccharomyces cerevisiae* (ASCA) was the first CD specific immune response thought to be targeted towards microbial antigens. IgA and IgG antibodies are directed against a specific oligomannosidic epitope present on the cell wall of the yeast saccharomyces[5]. To date it remains unknown as to what the specific microbial antigen ASCA is cross reacting with and giving rise to seropositivity specifically in the sera of patients with CD. ASCA is present in approximately 60% of CD patients, yet less than 5% in UC and non-IBD patients[6-8]. The specificity of ASCA renders a positive test result accurate in differentiating CD from UC and IBD from non-IBD in cases of diagnostic uncertainty. ASCA also remains an important marker of disease severity as defined by the development of complicating disease. More recent research has resulted in the identification of 3 additional markers representative of microbial driven immune responses, antibodies to the E.coli outer-membrane porin C (OmpC), the Pseudomonas fluorescens CD related protein (anti-CD related bacterial sequence {I2}) and the CBir1 flagellin. Antibodies to OmpC, whose antigen is purified from commensal *E.coli*[9,10], are present in 37%-55% of patients with CD and 2%-11% of patients with UC, while no more than 5% of non-IBD individuals express anti-OmpC[11-13]. I2 was isolated from affected colonic mucosa yet not in the unaffected segments and thus thought to be specific to CD[9]. Immune responses to this antigen are present in up to 55% of CD patients; however the specificity of this antibody has been questioned as it has been detected in the serum of UC patients (10%) and more impressively in up to 20% of non-IBD patients (9, data on file Prometheus Labs). Given its limitation as a diagnostic marker, it is unclear as to whether this immune response will provide additional prognostic information and further studies are needed. Serologic expression cloning was used to identify an immunodominant antigen, CBir1 flagellin, to which strong immune responses (B cell and CD4T cell) occurred in colitic mice[14]. These findings were translated from the bench to the bedside and approximately 50% of CD patients had serum reactivity to CBir1 whereas UC, inflammatory and healthy controls exhibited little to no reactivity to this flagellin[15].

All of the prior immune responses are specific to CD. In contrast perinuclear anti-neutrophil antibody (pANCA) is noted for its association with UC or a UC like phenotype. This IBD-specific ANCA displays a unique perinuclear highlighting (pANCA) on immunoflourence staining and is DNAse sensitive[16]. Although it remains undefined, it has been suggested that the antigen to which pANCA is directed is a nuclear histone (H1)[17]. This antigen is clearly distinct from the proteinase 3 or the myeloperoxidase reactivity observed in those patients with vasculitic disorders. pANCA is likely an autoantibody that is representative of a cross-reactivity with a luminal bacterial antigen[10,18,19]. Despite epidemiological and methodological differences, pANCA has been shown repeatedly to be prevalent in the sera of approximately 60% and 20% of UC and CD patients, respectively[6-8,20-23]. Typically, < 5% of non-IBD patients are pANCA positive.

Table 1 Pooled frequency of immune responses in CD, UC and non-IBD

Immune Response	%CD	% UC	% non-IBD
ASCA	60	< 5	< 5
Anti-OmpC	40	< 5	< 5
Anti-I2	54	< 10	20
Anti-CBir1	50	< 10	< 10
pANCA	20-25	65	< 5

As seen with the genetic and clinical heterogeneity of CD, studies have shown immune response (immune phenotype) heterogeneity exists among CD patients. Landers et al analyzed immune response heterogeneity in 330 patients and found that ASCA was detected in 56% of patients; 55% were seroreactive to OmpC 50% were seroreactive to I2, and 23% were pANCA[9]. Eighty-five percent responded to at least 1 antigen; only 4% responded to all 4. Among microbial antigens (ASCA, OmpC, I2), 78% responded to at least 1, and 57% were double positive, but only 26% responded to all 3. The level of response was stable over time and with change in disease activity. Among patients with the same qualitative antigen-response profiles, quantitative response differed. Moreover this study demonstrated that CD patients could be clustered into 4 distinct groups depending on their immune response patterns to microbial or autoantigens. One cluster was ASCA, a second was antibodies to OmpC and I2, the third pANCA and the fourth was low or no immune response to any tested antigens.

Subsequent analyses incorporating CBir1demonstrated that antibodies to CBir1 are present in approximately 40% of CD patients negative for antibodies to specific microbial antigens (ASCA, OmpC and I2) which suggests a unique immune phenotype[14]. Immune reactivity to CBir1 may further define CD phenotypes in that anti-CBir1 expression is present in 40%-44% of pANCA positive CD patients *vs* only 4% in pANCA positive UC patients. This difference may denote a unique etiopathogenic mechanism of disease that helps to further stratify patients based on immunogenetic phenotypes.

CLINICAL AND IMMUNE PHENOTYPES

Disease phenotype is not always a static phenomenon. Retrospective studies have examined the stability of disease phenotypes over the course of disease from time of diagnosis until point of last follow-up. It appears that disease location essentially remains stable over time, yet disease behavior evolves, such that after 20-yr of follow up, at least 80% of patients with originally non-complicating disease progress to complication, either

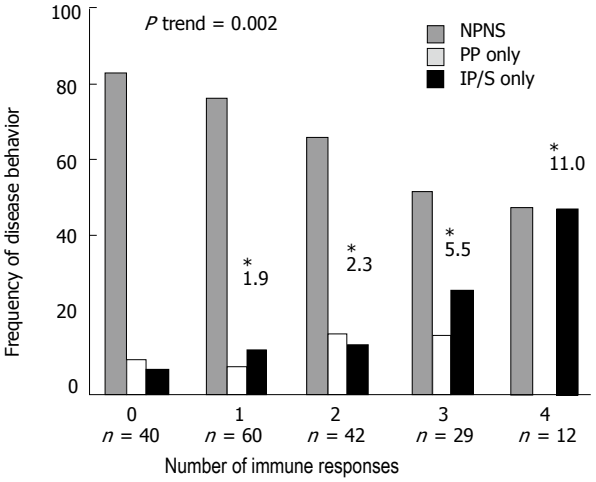


Figure 1 The frequency of disease behavior. The test for trend demonstrated a positive linear trend in the frequency of patients with IP and/or S disease as the number of positive immune responses toward I2, OmpC, ASCA and CBir1 increased (P = 0.002). The odds ratios (OR) reflect the odds of having internal penetrating and/or stricturing disease when positive for any 1, combination of 2, 3 or all 4 immune responses, as compared to those patients negative for all immune responses (baseline group).

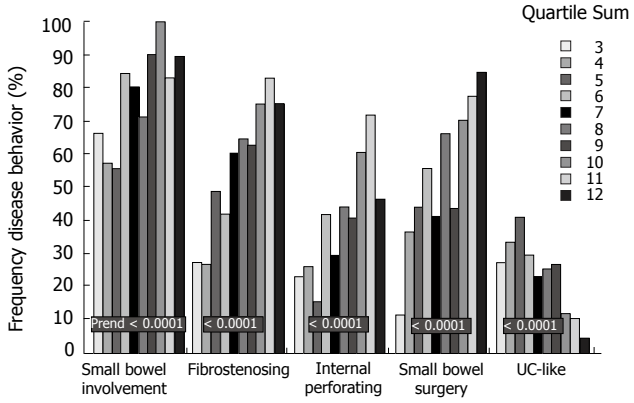


Figure 2 Association of the summed level of antibody responses towards multiple microbial antigens and disease phenotypes. Quartile sums used to reflect lowest (3) to highest titer level (12) for ASCA, anti-I2 and anti-OmpC.

penetrating or stricturing in nature[24-26].These findings suggest that non-complicating (non-penetrating, non-stricturing) disease behavior may not be a stable phenotype but just a temporary state that evolves in to one of the two complicating disease states over time.

Immune responses were first investigated as tools to differentiate UC from CD given the specificity of ASCA for CD and pANCA for UC. Advances in the sensitivity of the test characteristics lead to studies evaluating antibodies as diagnostic tools to differentiate IBD from non-IBD. Although conflicting, studies do support the use of these markers, particularly in children, to guide clinicians in cases of diagnostic uncertainty[8,27,28]. As new markers are identified and the test characteristics improve, the notion of immune responses optimizing diagnostic accuracy may become more clearer and clinically valid. It has become clear, however, that immune responses may also have perhaps a more important mechanistic implication in the pathogenesis of IBD. As alluded to above, these immune reactivities, as measured by the serological expression of immune responses to specific bacteria, may be representative of the host gene luminal bacterial interaction characteristic of IBD. Moreover if these immune responses represent the sum of a genetic and environmental predisposition to IBD, quantitative and qualitative expression of these immune responses may serve as an immunologic risk marker for IBD phenotypes. The initial immune-clinical phenotype studies demonstrated that although pANCA has been established as a UC-specific marker, approximately 25% of all CD patients also express pANCA. These CD patients are described as "UC-like" and tend to have an uncomplicated disease course[29-31]. In contrast, higher ASCA levels were shown to be associated with earlier age of disease onset, both stricturing and internal penetrating disease behaviors and need for small bowel surgery[30,31]. Further reports have found that patients with Crohn's disease who are positive for ASCA IgA, IgG, or both, may define a subset of patients with Crohn's disease at increased risk for early surgery[32]. ASCA has also been shown to be associated with a more aggressive disease course among a cohort of pediatric CD patients[33]. More recently anti-OmpC and anti-I2 have also been shown, like ASCA, to be associated with complicated disease behaviors among adult and pediatric CD patients[12,13,34]. Anti-CBir1 has also been shown to be independently associated with complicated disease behaviors in adult CD patients[14]. Of interest is that these associations were not found in a pediatric CD cohort suggesting potential differences in the mechanistic influence of CBir1 in the pediatric age group[34].

These studies also demonstrated that both the number of immune responses to the different microbial antigens expressed by a given individual as well as the magnitude (titer level) of these immune responses correlated most significantly with the presence of complicated CD phenotypes[12,34] (Figures 1 and 2). In theory, higher levels of immune reactivity may reflect the degree of loss of tolerance to specific microbial antigens rather than a global loss of tolerance. This may translate clinically to the magnitude of effect that specific bacteria have on the degree and extent inflammation resulting in more aggressive disease behaviors. Studies have demonstrated that immune responses to microbial antigens had a stronger association with the presence of complicated disease behaviors than known susceptibility genotypes, such that NOD2 was independently associated with small bowel disease location only and not disease behavior, fibrostenosing disease in particular, as previously reported[35-39]. None of these earlier phenotype correlation studies, however, took into account the assessment of multiple immune phenotypes.

IMMUNOGENETIC PHENOTYPES AND NATURAL HISTORY OF DISEASE

The prospective acquisition and determination of immune reactivity prior to the development of a disease complication is important to be able to truly evaluate the potential predictive value of immune responses in defining the natural history of IBD. It is generally agreed upon

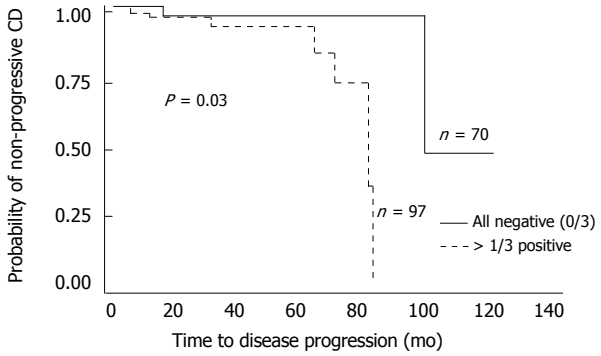


Figure 3 Kaplan Meier survival analysis. Comparison of time to progression from non-complicating to complicating disease behaviors between patients positive for 1 immune response to ASCA, I2 and OmpC (n = 97) (- - -) and those negative for all 3 (n = 70) (——).

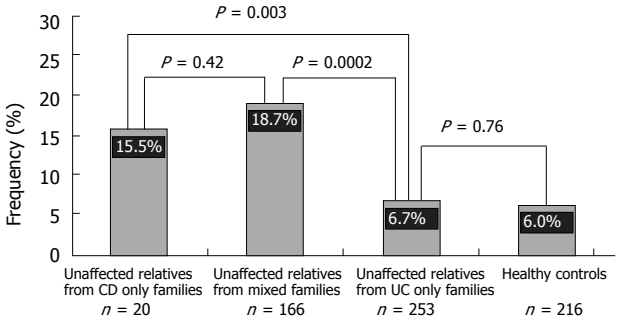


Figure 4 Frequency of anti-OmpC in unaffected relatives from CD only families, mixed families, UC only families, and healthy controls.

that the presence and level of immune responses do not change in a given CD patient[12,33]. We recently published the first prospective evaluation of the prognostic value of immune responses in patients presenting with non complicated CD[34]. This study included only those patients not presenting with internal penetrating and or stricturing disease at diagnosis and continued to be uncomplicated at the time sera was collected for immune response measurement so that it could be ascertained that the complication occurred after the antibodies were measured. Among those who developed a complication during the follow-up, the median time from diagnosis to the onset of the complication was 48 months. As of the last follow up of this cohort, 8% of the group seropositive to at least one serological marker had developed a complication *vs* only 3% in the seronegative group. Survival analysis (Figure 3) demonstrated that among those patients positive for at least one serology, more progressed to internal penetrating and or stricturing disease than those negative for all serologies. Saying it differently, those patients positive for at least one serology progressed faster that those negative for all serologies. Determining the factors that can predict the progression from uncomplicated to complicated disease states may stratify patients into at risk populations and impact the ultimate therapeutic management of patients with the goal of halting or more importantly preventing progression to complicated behaviors.

FAMILIAL EXPRESSION OF IMMUNE PHENOTYPES

There has been interest in evaluating whether immune responses are familial traits due to genetic factors. ANCA expression was the first marker to be studied. Several studies have observed an increased frequency of pANCA in unaffected relatives of UC patients but not environmental controls[40,41]. This relationship however has not been observed in all studies[42,43]. Sutton *et al* demonstrated that the quantitative and qualitative expression of ASCA was familial[44]. Another study confirmed that ASCA occur particularly frequently in CD patients, especially with the presence of a positive family history. However, they are also significantly increased in UC patients with a family history and in a considerable number of unaffected relatives of inflammatory bowel disease families, irrespective of the characteristics of their families (UC, CD, mixed, ASCA positive, and ASCA negative). The presence of ASCA in unaffected relatives might point towards a genetic predisposition to either CD or UC[45]. Studies in a twin population demonstrated an agreement in ASCA titers within concordant monozygotic twin pairs with Crohn's disease and suggested that the level of increase in ASCA reactivity(magnitude) is genetically determined[46]. None of these studies evaluated the influence of ASCA on the natural history of the affected offspring. A very recent study has shown that antibodies to OmpC have a strong familial aggregation pattern[47]. In this study, expression of anti-OmpC in unaffected family members of multiplex IBD families were compared with controls. As shown in figure 4, unaffected family members in CD only families had a similar frequency of anti-OmpC to those in mixed families. However, when comparing these two groups with healthy controls, both of them showed a much higher frequency of anti-OmpC expression. In addition, unaffected family members in these two groups displayed a greater prevalence of anti-OmpC than those unaffected from UC only families. This study also addressed whether ASCA and OmpC were co-segregating in patients and unaffected relatives. The results however suggest that anti-OmpC and ASCA may represent related but distinct immune responses in CD patients. More importantly the distinct immune phenotypes may translate into distinct clinical phenotypes.

SUMMARY

In summary, the emergence of immunogenetic phenotypes lends support to the proposed hypothesis that susceptibility genes regulate distinct immune processes, driven by luminal antigens, expressed as specific immune phenotypes which in turn influence clinical phenotypes in IBD patients. Immune responses to various microbial antigens among patients with CD may be related to different pathophyisological mechanisms of disease and a genetically susceptible host may yield distinct disease characteristics as a result of the immune responsiveness expressed by the at risk individual. Further research is necessary to validate the associations of immune responses to disease behavior and prognosis and for novel

immune responses to be identified so to provide more information on the underlying etiopathogenic mechanisms of characteristic of IBD. Advances in this field will afford clinicians the opportunity to create and implement appropriate and timely therapeutic management regimes based on the aggressiveness of the IBD subtype in order to alter and thus improve the long-term prognosis.

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TOPIC HIGHLIGHT

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Role of discs large homolog 5

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Abstract

In 2004, an association of genetic variation in the discs large homolog 5 (*DLG5*) gene with inflammatory bowel disease (IBD) was described in two large European study samples. The initial report of *DLG5* as a novel IBD susceptibility gene sparked a multitude of studies investigating its effect on CD and IBD, respectively, leading to controversial findings and ongoing discussions concerning the validity of the initial association finding and its role in the aetiology of Crohn disease. This review aims to summarize the current state of knowledge and to place the reported findings in the context of current concepts of complex diseases. This includes aspects of statistical power, phenotype differences and genetic heterogeneity between different populations as well as gene-gene and gene-environment interactions.

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Key words: DLG5; Association; Gender; Genetic heterogeneity; Inflammatory bowel disease; Crohn disease

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INTRODUCTION

In 2004, we described replicated association of genetic variation in the discs large homolog 5 (*DLG5*) gene with IBD and CD in two large European study samples consisting of German and UK patients[1]. The *DLG5* gene product is a member of the group of membrane-associated guanylate kinases (MAGUKs) and as such contains 4 PDZ domains, one SH3 domain followed by one guanylate kinase (GUK) domain. Additionally, this MAGUK contains an N-terminal domain of unknown function, DUF622. All of these domains are assumed to be involved in protein-protein interactions, supporting the notion of DLG5 as a multifunctional adapter and scaffold protein. DLG5 has been reported to be involved in maintaining cell shape and polarity[2] and to be located at sites of cell-cell contact[3]. The *DLG5* gene is located in a region of strong linkage disequilibrium which is independent of the surrounding genes due to substantial recombination. Analysis of the extended *DLG5* haplotype identified 4 common haplotypes, with haplotype A tagged by 8 htSNPs of equivalent genetic information content. While haplotype A was significantly undertransmitted in IBD and CD, haplotype D is significantly overtransmitted in both IBD ($\chi^2 = 8.08$, $P = 0.004$) and CD ($\chi^2 = 4.15$, $P = 0.04$). The only tagging SNP for haplotype D that allows discrimination from the underlying frequent haplotype C is the single nucleotide polymorphism 113G→A (reference SNP ID: rs1248696). The polymorphism results in the amino acid substitution R30Q in the DUF622 domain of DLG5. Comparisons with homolog proteins indicate that the R30Q variant may disturb binding to a Rab GTPase and thus is likely to have functional implications[1]. In addition, we described 5 additional rare, so-called 'private' mutations that are also likely to have functional implications but are running at a very low frequency in the general population and are thus unlikely to considerably contribute to the observed effect[1].

The initial report of *DLG5* as a novel IBD susceptibility gene sparked a multitude of studies investigating its effect on CD and IBD, respectively, unfortunately leading to discrepant findings and ongoing discussions concerning the validity of the initial association finding.

ROLE OF DLG5 IN JAPANESE PATIENTS WITH CROHN DISEASE

Shortly after the initial report, *DLG5* was analysed for its association to CD in Japanese patients[4]. The CD associated variant R30Q in individuals of Caucasian decent was absent in the Asian population. However, another variant in the *DLG5* gene, rs3758462, located in intron 15 of the *DLG5* gene, was significantly associated with CD ($P = 0.023$) in this Japanese population with an evaluated odds ratio (OR) of 1.39 confirming that *DLG5* is a susceptibility gene for CD with a moderate effect size. More important, the study by Yamazaki *et al* highlights the fact that ethnically divergent populations, although they share the same phenotype, need not necessarily share the same predisposing variants. One reason is that ethnically

divergent populations do not necessarily share the same risk genes as highlighted by the example of *CARD15* variants in ethnically divergent populations. While certain mutations in the *CARD15* gene, namely SNP8 (R702W), 12 (G908R) and 13 (1007insC), are strongly associated to CD in populations of Caucasian descent, the same mutations are completely absent in Asian populations. In fact, the *CARD15* gene is not even associated to the disease in Asian populations[5,6]; thus the finding that a variant in *DLG5* is - although modestly - associated with CD in a Japanese population implicates *DLG5* as a susceptibility gene that confers risk in populations of divergent ethnic origin. However, further studies involving additional cohorts of Asian or African origin will need to be studied to conclusively address the role of *DLG5* in ethnically divergent populations.

DLG5 AND HETEROGENEITY OF RISK VARIANTS IN POPULATIONS OF CAUCASIAN DESCENT

Daly *et al*[7] investigated the contribution of *DLG5* in three study cohorts consisting of patients from Quebec, Italy and UK. This study failed to replicate the reported undertransmission of haplotype A and suggested that the original observation could conceivably have been partially a statistical fluctuation and partially a result of LD with the observed R30Q association on haplotype D. However, they found evidence for an association of the R30Q variant with an estimated OR for R30Q of approximately 1.25, supporting the fact that *DLG5* is a moderate susceptibility gene in populations of Caucasian decent and weaker than in the initial report[1]. This finding implicates that if the true association was as modest as observed by Daly *et al* several thousand cases and controls would be required to have a > 90% power to replicate this effect with confidence ($P < 0.01$), an effort that can only be managed by a consortium effort as none of the individual investigators has a sufficiently powered study population at their disposal. Of note, Daly and colleagues[7] found evidence of replication of the *DLG5* association in only two of three of their sample collections, with the study sample from the UK failing to replicate the *DLG5* association. This observation pointed towards the possibility of either phenotypic differences between the populations studied or genetic heterogeneity within populations of Western European descent. The latter explanation was further supported by a more recent study which failed to replicate this association[8] in a Scottish population. In fact, in their study, the 113A allele was less common in IBD and CD patients than in healthy controls (11.4% in IBD *vs* 13.2% in healthy controls). The authors concluded that the observed discrepancies in allele frequencies might be explained by genetic heterogeneity within European populations. Such genetic heterogeneity has been observed even for the well established *CARD15* gene within Europe with Northern European populations exhibiting the lowest frequencies and Southern European populations exhibiting the highest frequencies of the variant alleles[9]. Accordingly, divergent association results for the R30Q variant in the *DLG5* gene may be indicative of the observed genetic heterogeneity in European populations. This finding is further corroborated by a recent study by Medici *et al*[10] who observed extreme heterogeneity for both *CARD15* and *DLG5* variants between a German and a Norwegian study sample, with a lower population attributable risk percentage for *CARD15* and *DLG5* variants in the Norwegian cohort than observed for other European cohorts. These results are consistent with an emerging pattern of a low frequency of e.g. *CARD15* variants in Northern countries where the prevalence of IBD is greatest. Interestingly, a recent study involving Greek patients with Crohn disease[11] showed that the R30Q variant was completely absent in their study population, further demonstrating the presence of extreme heterogeneity in *DLG5* variants between Northern and Southern European populations.

A recent meta analysis by Tenesa *et al*[12] combining the data from the studies reported by Stoll *et al*, Daly *et al* and Noble *et al* found substantial heterogeneity among the studies that reached statistical significance using the Cochran's Q statistic ($P = 0.0004$), and when including a fixed effect model, the R30Q variant was associated with an OR of 1.19 ($P = 0.035$)[12]. When further exploring the source of heterogeneity it became obvious that the frequencies among IBD samples tended to be more similar than between controls, an intriguing observation that will be further detailed in the following paragraphs.

ROLE OF GENDER

Environmental factors as well as gender may contribute to the complex basis of CD. While gender-specific gene effects are well documented in other complex diseases, i.e. cardiovascular diseases, gender has previously not been investigated in this context in inflammatory bowel disease. Interestingly, a follow-up study of the original genome-wide linkage study in German and UK IBD families[13] examined the data set for gender specific linkage[14] and identified the chromosomal region 10 q, harbouring *DLG5*, as a male specific CD susceptibility locus. In a recent study we investigated whether the association of the DLG5 variant R30Q to CD shows the same gender specificity as the underlying linkage signal. Using multivariate logistic regression analyses in a combined sample consisting of individuals from Germany, Italy and Quebec that were part of the studies reported by us[1] and Daly *et al*[7], we examined whether gender is a relevant factor to the association between CD and the R30Q variant in DLG5. Remarkably, our analyses revealed that the R30Q variant was associated with CD in men (OR = 2.49, $P < 0.001$) but not women (OR = 1.01, $P = 0.979$), thus establishing the DLG5 R30Q variant as a gender-specific susceptibility factor for CD[15]. This gender dependence may in part explain the divergent association studies relating to *DLG5* and IBD outlined in the paragraphs above, as gender composition of the cohorts at study may have been different, and gender was not included as an independent variable in previous analyses. Hopefully, reanalysis of the previous study data involving gender will help to further clarify the role of *DLG5* in the aetiology of IBD.

Of note, similar to the observation made by Tenesa

Table 1 Summary of genetic studies that analyzed DLG5 variants for their association to IBD or CD

	Study	Summary of results	Country of sample collection	Number of participants	Detailed results	Further sample info: gender/age
Association findings	Stoll *et al* 2004[1]	First finding of association between DLG5 variants and IBD/CD Positive association between R30Q (haplotype D tagging SNP) and IBD/CD Negative association between haplotype A and IBD/CD Positive association between P1371Q (rare haplotype) and IBD/CD Interaction between R30Q variant and CARD15 risk alleles	Germany	457 IBD trios (302 CD)	*TDT (T:U) single SNPs* R30Q IBD: 92:64, $P = 0.025$; CD: 60:42, $P = 0.075$ Haplotype A (DLG5_e26) IBD: 162:225, $P = 0.001$; CD: 108:151, $P = 0.008$ P1371Q IBD: 40:24, $P = 0.046$; CD: 24:17, NS	
			Germany, United Kingdom	1 Replication: 485 IBD trios (271 CD)	*TDT (T:U) single SNPs* R30Q IBD: 90:73, $P = 0.09$; CD: 58:43, $P = 0.065$ Haplotype A (DLG5_e26) IBD: 165:214, $P = 0.006$	
			Northern Europe	2 Replication: 538 CD patients, 548 controls	*Allele frequencies (cases versus controls)* R30Q CD: 13.2 %, controls: 9%, OR = 1.62, $P = 0.001$ HaplotypeA CD: 32.4 %, controls: 36.1%, OR=0.74, $P = 0.01$ P1371Q CD: 4.2 %, controls: 2.5%, OR=1.51, $P = 0.01$	Controls age and sex matched
	Daly *et al* 2005[7]	Replication of positive association between R30Q and CD in two of three independent study samples No replication of negative association between haplotype A and CD P1371Q variant not polymorphic	Canada, Italy	249 CD patients, 207 controls	*Allele frequencies (cases versus controls)* R30Q CD: 11%, controls: 5.9%, $\chi^2 = 7.8$, $P = 0.003$ Haplotype A CD: 36.5%, controls: 40.1 %, $\chi^2 = 1.4$, $P = 0.12$ P1371Q Not polymorphic	
			United Kingdom	353 CD patients, 336 UC patients, 493 controls	*Allele frequencies (cases versus controls)* R30Q CD: 9.3%, controls: 9.7%, NS Haplotype A CD: 34%, controls: 35.7%, NS	
			Canada, United Kingdom	306 IBD trios (88 % CD)	*TDT (observed:expected transmissions)* R30Q IBD: 76:65.1, $P = 0.018$ Haplotype A IBD: 304: 307.8, NS	
	Newman *et al* 2006[25]	Non replication of positive association between R30Q variant and IBD/CD Association between haplotype A and IBD/CD, however positive association in this study instead of negative Replication of positive association between P1371Q and IBD/CD	Canada	402 CD patients, 179 UC patients, 537 controls	*Allele frequencies (cases versus controls)* R30Q Controls: 8.7 %; IBD: 8.2 %, NS; CD: 7.8 %, NS Haplotype A Controls: 30.8 %; IBD: 36.1 %, $P = 0.01$; CD: 36.6 %, $P = 0.01$ P1371Q Controls: 6.1 %; IBD: 9.6%, $P = 0.01$; CD: 10.3%, $P = 0.01$ Frequencies for both variants did not differ between males and females in the cases and controls	Mean age CD patients: 20.4 yr Controls: 31 yr
Non replication findings	Török *et al* 2005[26]	Non replication of positive association between R30Q and IBD/CD Non replication of negative association between haplotype A and IBD/CD Non replication of positive association between P1371Q and IBD/CD Non replication of interaction between R30Q variant and CARD15 risk alleles	Southern Germany	625 CD patients, 363 UC patients, 1012 controls	*Allele frequencies (cases versus controls)* R30Q Controls: 10.8%; IBD: 10.1%, NS; CD: 9.8%, NS Haplotype A Controls: 35.2%; IBD: 34.4%, NS; CD: 34.6%, NS P1371Q Controls: 4.4%; IBD: 4.5%, NS; CD: 3.9%, NS	Controls age and sex matched
	Noble *et al* 2006[8]	Non replication of positive association between R30Q and IBD/CD Non replication of negative association between haplotype A and IBD/CD Non replication of interaction between R30Q variant and CARD15 risk alleles	Scotland	374 CD patients, 305 UC patients, 294 controls	*Allele frequencies (cases versus controls)* R30Q Controls: 13.2%; IBD: 11.4%, P = NS; CD: 11.4 %, NS Haplotype A Controls: 31.5 %; IBD: 35 %, $P = 0.17$; CD: 36.9 %, $P = 0.078$	Numbers Male/female (median age) CD patients: 181/193 (28 yr) Controls: 143/151 (39 yr)
	Vermeire *et al* 2005[24]	Non replication of positive association between R30Q and IBD/CD Non replication of negative association between haplotype A and IBD/CD Replication of interaction between R30Q variant and CARD15 risk alleles	Belgium	373 IBD trios (80 % CD)	*TDT (T:U) single SNPs* R30Q IBD: 51:79, $P = 0.01$; CD: 42:71, $P = 0.01$ Haplotype A (DLG5_e26) IBD: 161:149, NS; CD: 134:123, NS	Numbers Male/female (mean age) IBD patients: 162/211 (37 yr)
				472 CD patients, 120 UC patients, 305 controls	*Allele frequencies (cases versus controls)* R30Q Controls: 10.8%; IBD: 11.8%, NS; CD: 11.5%, NS Haplotype A Controls: 33.8 %; IBD: 33.9 %, NS; CD: 35.2 %, NS	IBD patients: 256/352 (45 yr) Controls: 139/166 (41 yr)

R30Q not polymorphic	Yamazaki *et al* 2004[4]	R30Q polymorphism not polymorphic in Japanese patients Non replication of negative association between haplotype A and CD Non replication of positive association between P1371Q and CD Other DLG5 variant risk associated instead	Japan	484 CD patients, 345 controls	*Allele frequencies (cases versus controls)* R30Q Polymorphism absent in 48 Japanese patients tested Haplotype A CD: 24.6 %, controls: 22%, NS P1371Q CD: 16.5%, controls: 19.2%, NS DLG5 SNP rs3758462 CD: 18.5 %, controls: 22.1%, $P = 0.068$ ($P = 0.023$, additive inheritance model for rare variant)	
	Gazouli *et al* 2005[11]	R30Q polymorphism absent in Greek study sample	Greece	120 CD patients, 85 UC patients, 100 controls	R30Q Polymorphism absent	
R30Q gender specificity	Friedrichs *et al* 2006[15]	Gender specific analysis of R30Q polymorphism in German, Italian and Canadian study samples with positive association findings[1,7] R30Q confers susceptibility to CD in males but not females Observation of possible transmission ratio distortion (TRD) in general population TRD confirmed by two independent population based samples, one of which was a birth cohort	Germany, Italy, Canada	613 CD patients, 749 controls	*Allele frequencies (cases versus controls)* R30Q Q allele frequencies male CD patients: 10.1%, male controls: 5.2% $OR_{males} = 2.5$, 95% confidence interval = 1.5-4.1, $P < 0.001$ female CD patients: 10.9%, female controls: 11.3% $OR_{females} = 1$, NS TRD Q allele frequencies newborn study sample males: 7.1%, females: 11%	Numbers Male/female (mean age) CD patients: 235/375 (36 yr) Controls: 403/346 (40 yr)

TDT: Transmission disequilibrium test; T:U: Transmitted alleles versus untransmitted alleles (transmissions from heterozygous parents to affected offspring); OR : Odds ratio; NS: Not significant.

et al[12], the observed difference for men and women is result of divergent allele frequencies in the healthy controls and not in the CD patients. The allele frequencies for the Q allele were almost identical among men and women affected with CD (CD males: 10.1%, CD females 10.9%) while there was a significant difference in healthy individuals (males controls: 5.2 %, female controls: 11.3%). These data suggest gender-specific genetic heterogeneity or, more likely, the presence of transmission ratio distortion (TRD) of the Q allele among healthy controls. This hypothesis was further supported by two independent population-based study samples, where similar observations were made[15]. Interestingly, one of the two study samples consisted of a study sample of newborns (Q allele: 7.1% in males *vs* 11% in females, $P = 0.036$) indicating that the observed gender-specific disparity of Q allele frequencies is in fact consequence of prenatal processes, and likely of gender-dependent transmission distortion. However, other mechanisms or a combination of all possible causes for TRD, i.e. biased meiotic segregation, gametic selection and viability selection are conceivable and the true underlying mechanisms are subject to further research. The question remains why the observed gender-dependent disparity in Q allele frequencies is offset in male CD patients. In our opinion, the increase in Q alleles in affected individuals could be either consequence of a yet unknown, disease related mechanism or result of an accumulation of the risk associated Q allele in affected men but not women. However, further studies will need to be conducted to conclusively address this question.

ROLE OF GENE-GENE INTERACTION

Prior to the identification of *DLG5* as a susceptibility gene for IBD and CD, respectively, two CD risk genes were identified, *CARD15* and the *IBD5* risk haplotype that since then have been unequivocally replicated several times[16-23]. As IBD is a polygenic, multifactorial disease, the potential gene-gene interaction between the known susceptibility variants in *CARD15, IBD5* and *DLG5* needs to be investigated to estimate the overall genetic risk. However, most of the studies on IBD genetics are hampered by the lack of statistical power to address gene-gene interactions due to small sample size, rendering such studies a challenging endeavour. This is particularly true for *DLG5*, as *DLG5* is a susceptibility gene that only confers a modest risk[7], the DLG5 R30Q risk variant is comparably infrequent (5%-10%) and subject to genetic heterogeneity[12]. Despite the before mentioned restrictions in the investigation of gene-gene interactions, we examined potential locus-locus interactions with variants of *CARD15* in our initial study describing genetic variation in DLG5[1]. Interactions between *DLG5* and *CARD15* were examined by stratifying trios in two groups on the basis of the *CARD15* genotype of the affected child. Interestingly, in CD trios, we observed a significant difference in transmissions of R30Q between the $CARD15^{risk}$ and $CARD15^{nonrisk}$ group with an increased association of the risk allele in the $CARD15^{risk}$ group, while the association was no longer seen in the $CARD15^{nonrisk}$ group. This suggested a potential interaction of the DLG5 R30Q variant with $CARD15^{risk}$ variants. Recently, Vermeire *et al*[24] reported that *DLG5* was not associated in a large Flemish IBD cohort when *DLG5* was analyzed without considering the genotype status at *CARD15*[24] variants. However, after stratification for $CARD15^{risk}$ variants in the CD patients, the R30Q frequency turned out to differ significantly in the $CARD15^{risk}$ group (Q frequency 12.2%) compared to the $CARD15^{nonrisk}$ group (Q frequency 8.7%; $P = 0.033$), providing further evidence for a potential interaction between $CARD15^{risk}$ variants and DLG5 R30Q. However, further studies involving large study samples will need to be conducted, although due to the observed genetic heterogeneity in *DLG5* variation even within Europe, a consortium effort will be difficult to conduct, and the results are likely difficult to interpret. (Table 1).

In summary, genetic variation in *DLG5* and its role in IBD is a prime example for the difficulties encountered in the dissection of the genetic basis of complex disease. Similar to many examples from other complex diseases, the primary association signal could not be unequivocally replicated in other independent study populations. However, with more and more studies emerging and the growing awareness about the complexity of multifactorial diseases in general, the picture appears to clear off - although there are still a lot of loose ends to tie. The example of *DLG5* shows for the first time that gender needs to be considered as an independent factor contributing to the genetic risk for IBD, a fact that has not been considered previously as the prevalence of IBD is almost equal among genders.

In order to further elucidate the contribution of present and emerging susceptibility genes to the aetiology and clinical presentation of IBD, there is the need to have well-characterized and homogeneous patient populations as well as appropriate controls, preferentially with standardized phenotypes to allow for comparisons between independent study cohorts. In addition, investigators need to be aware of the extreme heterogeneity in IBD disease variants among European populations and between ethnically divergent populations. Finally, the investigation of gene-gene and gene-environment interaction and its influence on disease-onset and progression will gain more importance. Due to the availability of whole genome genotyping arrays, more susceptibility variants will become available in the near future, all of which require further validation in the context of existing IBD susceptibility genes and environmental factors.

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TOPIC HIGHLIGHT

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Pharmacogenetics in inflammatory bowel disease

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Abstract

Pharmacogenetics is the study of the association between variability in drug response and (or) drug toxicity and polymorphisms in genes. The goal of this field of science is to adapt drugs to a patient's specific genetic background and therefore make them more efficacious and safe. In this article we describe the variants in genes that influence either the efficacy or toxicity of common drugs used in the treatment of inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn's disease (CD) including sulfasalazine and mesalazine, azathioprine (AZA) and 6-mercaptopurine (6-MP), methotrexate (MTX), glucocorticosteroids (CSs) and infliximab. Furthermore, difficulties with pharmacogenetic studies in general and more specifically in IBD are described. Although pharmacogenetics is a promising field that already contributed to a better understanding of some of the underlying mechanisms of action of drugs used in IBD, the only discovery translated until now into daily practice is the relation between thiopurine S-methyltransferase (TPMT) gene polymorphisms and hematological toxicity of thiopurine treatment. In the future it is necessary to organize studies in well characterized patient cohorts who have been uniformly treated and systematically evaluated in order to quantitate drug response more objectively. An effort should be made to collect genomic DNA from all patients enrolled in clinical drug trials after appropriate informed consent for pharmacogenetic studies.

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Key words: Inflammatory bowel disease; Pharmacogenetics; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic invalidating relapsing and remitting diseases of the bowel. Crohn's disease (CD) and ulcerative colitis (UC) are the two main clinical presentations. Within these subgroups there are many differences regarding disease extension, localization, behavior and the occurrence of extra-intestinal manifestations (EIM)[1,2]. The precize ethiology of IBD is unknown but bot environmental factors and a genetic susceptibility play a role. Although great advances have been made in the management of the disease with the introduction of immune-modulators and monoclonal antibodies, a curative therapy does not yet exist. Mesalazine or sulfasalazine formulations are the first-line drugs for induction and maintenance of remission in UC. Glucocorticosteroids (CSs) are added if necessary[3]. For severe acute UC cyclosporine is an option if intravenous steroids fail to induce remission [4]. Azathioprine (AZA) and 6-mercaptopurine (6-MP) are efficacious to maintain remission although the evidence is rather weak. Recently the ACT1 and ACT2 trials proved the usefulness of chimeric monoclonal antibodies to TNF-alpha (TNF-α) (infliximab) for induction of remission and maintenance therapy of therapy refractory UC[5,6]. Active CD is treated with steroids and (or) antibiotics. AZA, 6-MP or methotrexate (MTX) are added for maintenance of remission[3,7]. The role of sulfasalazine or aminosalicylates in the induction and maintenance of medically induced remission of CD is limited[8]. Infliximab has become the treatment of choice for both induction and maintenance of remission in refractory luminal and fistulizing CD[9]. A great inter-patient variability exists in efficacy and toxicity of the drugs used. There are many factors that influence response to pharmaco-therapy including disease severity and complications, environmental factors such as smoking and genetic factors. Variability in drug response is greater across a population than within the same patient or in monozygotic twins, therefore part of this difference is attributed to genetic factors. It is estimated that polymorphisms in genes can account for 20%-95% of variability in drug effects[10]. The scientific

domain studying the relation between genetic variability and variability in drug response and toxicity is called pharmacogenetics[11]. Primary candidate genes of interest include those encoding for drug receptors, metabolizing enzymes, and transporters. However, selection of optimal drug therapy may also involve disease susceptibility genes indirectly affecting drug response. Pharmacogenetics holds the promise that drugs might one day be adapted to each person's own genetic background and therefore be more efficacious and safe. In this article we describe (1) the genetic variants in genes that influence the toxicity of respectively AZA and 6-MP, sulphasalazine and mesalazine and MTX and (2) polymorphisms in genes that influence response to GCs and infliximab. Finally we will discuss the clinical usefulness of and the problems associated with pharmacogenetic studies in IBD.

VARIABILITY IN TOXICITY

The definition and diagnosis of side effects is usually less complicated than the definition of response to therapy although there are confounding factors such as the use of multiple drugs simultaneously. A study that compares the prevalence of side effects between 2 groups of patients with and without a polymorphism is relatively easy to perform. The genetic polymorphisms in the thiopurine S-Methyltransferase (TPMT) gene and its influence on AZA and 6-MP treatment is the first example of how genotyping can be used to optimize drug therapy in IBD. We also describe the relation between polymorphism in N-acetyltransferase genes (NAT) and side effects of mesalazine and sulphasalazine and variants in genes encoding MTX metabolizing enzymes.

Azathioprine and 6-mercaptopurine

Efficacy and toxicity of the drugs: Indications for the use of 6-MP or the prodrug AZA in moderate to severe CD are maintenance of remission and steroid sparing[12,13]. They are used in the treatment of fistulizing CD and recommended during infliximab treatment to reduce antibody formation and to optimize response rates[14]. The drugs are also useful in maintenance therapy of UC[15]. Approximately 40% of IBD patients do not respond to AZA therapy[12,13,15]. Ten to twenty-five percent of patients have to withdraw from AZA or 6-MP due to major (leucopenia, pancreatitis, infection and malignancy) or minor (rash, nausea, fever, arthralgias, malaise and diarrhea) adverse events[16]. Myelosuppression is dose dependent and can be lethal and occurs in 1% to 5% of the patients[12,13,17,18].

Metabolization and pharmacogenetics: AZA is a prodrug that is administered orally at a daily dose of 2.5 mg/kg. Sixteen percent to 50% of the drug are absorbed in the gut. AZA is broken down by non-enzymatic degradation to 6-MP and methyl-nitrothioimidazole. 6-MP can also be administered directly and the current dose is 1 to 1.5 mg/kg. 6-MP is converted to the nucleotide 6-thioinosine monophosphate (6-TIMP) by the enzyme hypoxanthine guanine phosphoribosyl-transferase (HGPRT). 6-TIMP is further metabolized to thioguanine mono-, di- and triphosphates [thioguanine nucleotides (TGNs)]. Alternatively 6-MP can be inactivated by xanthine oxidase (XO) into 6-thiouric acid (6-TU) or by TPMT into 6-methylmercaptopurine (6-MMP). TPMT also catalyses the methylation of the nucleotide metabolites including 6-tIMP and thioguanosine 5-monophosphate[19] (Figure 1). The relative activities of the TPMT, XO and HGPRT enzymes determine the amount of the active 6-TGN and other 6-MP metabolites. The precise mechanism of action of the drug is not completely understood. Incorporation of TGNs in DNA plays a role in immune suppression and bone marrow toxicity[20]. More recent studies suggest that TGNs may also act by inhibition of Rac1, a GTP binding protein involved in the CD28 signal transduction pathway in T cells. CD28 co-stimulation is essential for T cell activation. Through inhibition of Rac1, its target genes [e.g. mitogen-activated protein kinases, NF-kB, bcl-x(L)] are suppressed[21]. There is also evidence that methyl TIMPs inhibit the de novo purine synthesis[22].

TPMT activity: In 1980 Weinshilboum *et al* described for the first time inherited differences in TPMT activity[23]. In the mean time 2 wildtype TPMT alleles (TPMT*1 and TPMT*1S) and 16 variant alleles with low enzymatic activity (TPMT*2, *3A, *3B, *3C, *3D, *4-15) have been described[24-28]. In the Caucasian and African populations approximately 90% of individuals carry 2 wildtype alleles resulting in high TPMT enzyme activity, 10% are heterozygous and display intermediate activity and 0.3% are homozygous for low activity alleles and display no detectable TPMT activity. Heterozygosity is less frequent in Asians (2%-5%). The TPMT*2, *3A and *3C alleles account for 80% to 95% of inherited TPMT deficiency in different populations over the world[29-31]. The TPMT*2 allele contains a non-synonymous mutation at amino acid position 80 (Ala80Pro, G238C)[32]. The TPMT*3A allele contains two missense mutations, G460A (Ala145Thr) and A719G (Tyr240Cys). The TPMT*3C allele contains only the A719G missense mutation[33]. In African, African-American and Asian populations the most common low TPMT activity allele is the TPMT*3C and in Caucasians the TPMT*3A allele. The TPMT *3B (G460A) allele occurs in 1% of Caucasians. This allele is however important because PCR based assays can not distinguish between TPMT 1/*3B and *3B/*3C genotypes[34] (Table1).

TPMT genotype and hematopoietic side effects: A hundred percent of the compound heterozygous or homozygous IBD patients develop severe hematotoxicity when treated with a normal thiopurine dose[35-38]. The inverse is not entirely true as Colombel *et al* found that only 27% (11/41) of IBD patients treated with AZA who developed myelosuppression had one or more of the 3 frequent variant alleles[39]. However, in a study in patients treated with AZA for rheumatoid arthritis(RA) by Black *et al* 5 of 6 (83%) patients with leukopenia were heterozygous for a TPMT variant allele[40,41]. Some investigators suggest that thiopurine drugs should entirely be avoided in patients with low TPMT activity (heterozygous), however it seems that IBD patients with low TPMT activity (heterozygous) can mostly safely be treated with reduced doses of AZA or 6-MP provided they are submitted to close monitoring[42,43]. Homozygous or compound heterozygous patients with no TPMT activity should not receive the drug.

Table 1 Interpretation by PCR based methods of TPMT activity

Genotype		PCR	Risk of hematopoietic toxicity
238G/238G	*1/*1	238 GG	-7%
460G/460G		460 GG	
719A/719A		719 AA	
238G/238G	*1/*3A	238GG	-35%
460A/460A		460GA	
719A/719G		719AG	
238G/238G	*1/*3B	238GG	-35%
460G/460A		460GA	
719A/719A		719AA	
238G/238G	*1/*3C	238GG	-35%
460G/460G		460GG	
719A/719G		719AG	
238G/238G	*3B/*3C	238GG	-100%
460G/460G		460GG	
719A/719G		719AG	
238G/238G	*3A/*3C	238GG	-100%
460A/460G		460GA	
719G/719G		719GG	
238G/238G	*3A/*3B	238GG	-100%
460A/460A		460AA	
719G/719A		719AG	

Patients with *1/3A and *3A/*3C genotype have the same PCR results, however the risk to develop hematopoietic toxicity for patients with 2 low activity alleles is almost 100% compared to 35% only for heterozygous patients.

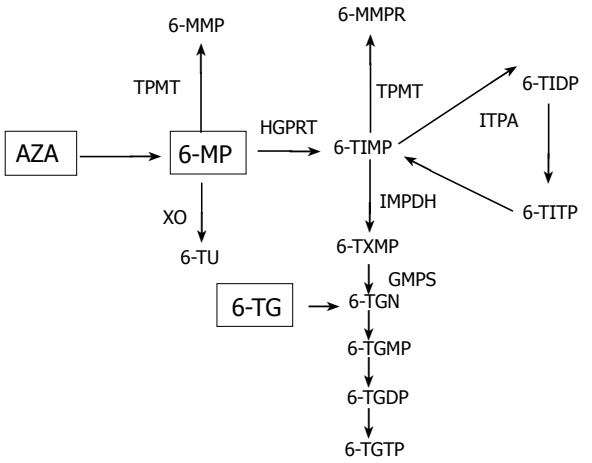


Figure 1 Simplified scheme of AZA metabolism: AZA is non-enzymatically converted to 6-MP. 6-MP is converted into the 6-thioinosine monophosphate (6-TIMP) by the enzyme hypoxanthine guanine phosphoribosyl-transferase (HGPRT). 6-TIMP is further metabolized to thioguanine mono, di and triphosphates [6-thioguanine nucleotides (TGN)]. Alternatively 6-MP can be inactivated by xanthine oxidase (XO) into 6-thiouric acid (6-TU) or by TPMT into 6-methylmercaptopurine (6-MMP). TPMT also catalyses the methylation of the nucleotide metabolites including 6-TIMP and 6-thioguanosine-5'-monophosphate to 6-methylmercaptopurine (6-MMPR). Inosine monophosphate dehydrogenase (IMPDH) and guanine monophosphate synthetase (GMPS). Inosine triphosphate pyrophosphatase (ITPA), 6-thioinosine triphosphate pyrophosphate (6-TITP).

TPMT activity and other side effects: One study has described a relation between low TPMT activity and nausea, however this has not been replicated[44]. More recently a relation between therapy refractoriness and normal or high TPMT activity has been described. Predicting response is useful especially since there is a 3 month delay before the effect of these drugs is achieved. Efficacy of treatment with 6-MP or AZA is related to red blood cell levels of the active metabolite TGN (> 235 pmol/8×10^8 RBC)[38]. Patients heterozygous for TPMT low activity alleles have lower TPMT activity and higher erythrocyte TGN levels[45] (Figure 1). One study showed a lower relapse rate in patients with low TPMT activity compared to patients with normal TPMT activity but 2 later studies failed to replicate this finding[45-47]. High levels of 6-MMP are thought to be responsible for hepatotoxicity[48]. Liver transaminase elevation has been associated with high TPMT activity and 6-MMP levels and TPMT variant alleles are very rare in patients with hepatotoxicity[48]. This finding has not been replicated.

TPMT activity from bench to bedside: Genotyping the 3 most common SNPs or measuring the enzyme activity are 2 alternative methods for predicting the risk for hematopoietic toxicity. TPMT enzymatic activity can be measured in red blood cells with a radiochemical or high-performance liquid chromatography assay, however the results are modified by recent blood transfusions[49]. TPMT activity also varies over time and is influenced by other drugs (e.g. diuretics, 5-ASA) and conditions (e.g. uremia)[50-52]. TPMT enzyme activity will identify patients with high TPMT activity that metabolize 6-MP to 6-MMP and therefore may be resistant to treatment with thiopurine drugs[48]. As described PCR based assays can not distinguish between the TPMT 1/*3B and *3B/*3C genotypes[34]. Furthermore there are problems with the correlation between enzyme activity assays and genotype, since patients with low-normal enzyme activity can have either a wildtype or a heterozygous genotype[41,55].

There is no consensus yet that TPMT genotype or phenotype should be measured before starting AZA or 6-MP treatment in IBD patients, especially since hematologic toxicity can develop in patients with normal TPMT activity. Monitoring of blood counts and liver transaminases remains therefore necessary in all patients. If TPMT enzyme activity is measured patients with normal TPMT activity (or wildtype patients) can receive 2-2.5 mg/kg AZA or 1-1.5 mg/kg of 6-MP. Patients with intermediate activity (heterozygous) should have a dose reduction of 50% and patients with low or absent TPMT activity (compound heterozygous or homozygous) should not receive thiopurine drugs at all or at very low dose (10%)[54]. One socio-economic study in IBD has proven that TPMT phenotyping or genotyping is cost effective to identify patients with low or no TPMT enzyme activity (homozygous or compound heterozygous) in order not to treat them and avoid severe hematological complications[55].

ITPA genotype and thiopurine toxicity: Inosine triphosphate pyrophosphatase (ITPA) deficiency results in the accumulation of the inosine nucleotide ITP and a methylate product (Figure 1). Sixty-two IBD patients suffering adverse drug reactions to AZA therapy and 68 AZA treated patients without side effects were genotyped for the ITPA SNP C94A by Mariniki *et al*[44]. The ITPA C94A deficiency-associated allele was significantly associated with flu-like symptoms (OR 4.7, 95% CI

1.2-18.1, $P = 0.03$), rash (OR 10.3, 95% CI 4.7-62.9, $P = 0.02$) and pancreatitis (OR 6.2, CI 1.1-32.6, $P = 0.05$). This finding was not replicated in two subsequent studies in 72 and 41 patients respectively[56,57]. The thiopurine drug 6-thioguanine (6-TG) is not metabolized by TPMT and ITPA and might therefore be efficacious for patients with AZA or 6-MP resistance and high TPMT activity or for patients with impaired ITPA activity (Figure 1). However 6-TG treatment is associated with an increased risk of nodular regenerative hyperplasia of the liver and veno-occlusive disease[58,59].

Sulphasalazine and mesalazine

Variability in drug acetylation was discovered 45 years ago in patients who were treated with isoniazide for tuberculosis. Family studies revealed that this variation was genetically controlled and that the population can be divided into slow acetylators and rapid acetylators due to polymorphisms in the N-acetyltransferase genes (NAT). Two iso-enzymes NAT1 and NAT2 that have overlapping and specific aryl-amine substrates have been identified in humans[60]. Eleven single nucleotide polymorphisms (SNPs) in the NAT2 gene result in 25 different alleles in humans[61]. More than 50% of Caucasians are NAT2 slow acetylators[62]. The slow acetylating phenotype for NAT2 (reduction of 10% to 20% of the cytosolic NAT2 protein) is a heterozygous trait and several low activity alleles have been described (Table 2)[61,63,64]. There is also an allele (NAT2*12B) associated with high NAT2 expression and a fast acetylating phenotype (Table 2). Lin *et al* found that 4 variants (191A, 481T, 590A and 857A) accounted for nearly all slow acetylating phenotypes in Caucasian, African, Hispanic and Asian populations[65]. A polymorphism in the NAT1 gene was first described in 1993 and since over 20 alleles have been identified[63,66,67]. Several variant alleles are functional and result in reduced N and/or O-acetylation in humans (NAT*14A, NAT1*14B, NAT1*17, NAT1*19 and NAT1*22) and yeast cells (NAT1*14B, NAT1*15, NAT1*17, NAT1*19 and NAT1*22)[66,68,69]. Sulphasalazine is still widely used for long term maintenance therapy in UC. Five percent to fifty-five percent of the patients treated with the drug develop hypersensitivity or intolerance. Sulphasalazine is split in the lumen of the colon into the active metabolite 5-ASA and sulphapyridine (SLP) by bacterial azo-reductases. SLP is absorbed and acetylated in the liver by NAT2 into N-acetyl-SLP. Levels of SLP above 50 mg/L are associated with most of the severe side effects (thrombocytopenia, leukopenia, liver and lung toxicity and pancreatitis) and are observed more frequently in slow acetylators[70]. Case reports suggested a relation between side effects like leukopenia and a mononucleosis-like syndrome and one of the slow acetylating genotypes in IBD patients treated with sulphasalazine[71,72]. Mesalazine exists in different targeted release preparations designed to protect 5-ASA from absorption and metabolism in the proximal digestive tract. 5-ASA is released in the distal ileum or the colon and part is systemically absorbed. 5-ASA is acetylated in the liver by NAT1 into N-acetyl 5-ASA (N-Ac-5ASA) and excreted in the urine. Side effects are less common with mesalazine compared to sulphasalazine but there is still some concern about nephrotoxicity with long term use. A retrospective study in 77 UC patients did not show an impact of NAT1 genotype on efficacy or side effects of mesalazine [73].

Table 2 NAT2 phenotypes and genotypes

Mutations	Allele designation	Phenotype
None	NAT2*4 (wild-type)	normal
T341C, C481T, A803G	NAT2*5B (r3,S1a)	Slow
C282T, G590A	NAT2*6A (M2)	Slow
T341C, C481T	NAT2*5A (M1)	Slow
T341C, CA803G	NAT2*5C (S1c)	Slow
C282T, G857A	NAT2*7B (M3)	Slow
C282T	NAT2*13 (S4)	
G590A	NAT2*6B (R2)	Unknown
G191A	NAT2*14A (M4)	Slow
A803G	NAT2*12A	Rapid

Methotrexate

MTX is efficacious for the induction of remission while weaning patients from steroids in 39% of CD patients and for maintenance of remission in 65% of CD patients after successful induction therapy with this drug[7]. A low single weekly dose of 15 to 25 mg intramuscularly or subcutaneously is used. Most pharmacogenetic studies on MTX are derived from the oncologic literature where patients receive a high dose MTX. MTX is taken up by the cell trough reduced folate carrier-1 (RFC1) and polyglutaminated by folyglutamate synthetase (FPGS)[74]. MTX polyglutamate inhibits amino-imidazole carboxamide (AICAR) transformylase, an enzyme of the de novo purine synthesis pathway. MTX also inhibits the enzyme 5, 10-methylenetetrahydrofolate reductase (MTHR) that catalyses the conversion of homocystein to methionine (Figure 2). Deficiency of MTHR and hyperhomocysteïnemia are implicated in neurological and vascular diseases[75]. Inhibition of MTHR and DNA synthesis is the primary mechanism of action of MTX in oncology. Tissues undergoing rapid cell turnover with a high fraction of the cell in the S-phase of the cell cycle are the most susceptible to its cytocidal effects. This mechanism also explains the possible severe side effects of MTX including bone marrow suppression, mucositis, gastro-intestinal toxicity and liver function abnormalities. Folic acid or folinic acid supplementation diminishes the rate of these side effects[76]. The common C677T SNP in the MTHFR gene is associated with decreased MTHFR activity and altered folate levels and has been studied extensively[77]. The 677TT genotype has been identified as a risk factor for elevated ALT values in MTX treated RA patients[77]. In patients undergoing bone marrow transplantation patients homozygous for the 677T allele had more often oral mucositis and platelet recovery was slower[78,79]. In patients with breast cancer and MTX chemotherapy neutropenia was more frequent in patients with the TT genotype[80]. The risk of side effects for patients with the TT genotype was reduced with folic acid supplementation in RA patients[76]. A second SNP in the 3'-untranslated region of the MTHFR gene is associated with higher expression

of the gene[81]. No studies are available that examine the relation between these variants and the prevalence of side effects in IBD patients treated with low dose MTX. The exact anti-inflammatory mechanism of low dose MTX therapy in RA and IBD is not clearly known. In contrast to side effects the anti-inflammatory effect is not inhibited by folic acid supplementation[76]. A possible mechanism of action is inhibition of AICAR and a subsequent rise in intracellular adenosine[74]. Polymorphisms in RFC1, thymidilate synthetase, and in AICAR transformylase have been implicated in response to therapy with low dose MTX in RA[82]. Polymorphisms in DHFR, in enzymes involved in catabolism of MTX (γ-glutamyl hydrolase) or its efflux have also been suggested to play a role in response to treatment. No data on the relation between any of these SNPs and IBD are available.

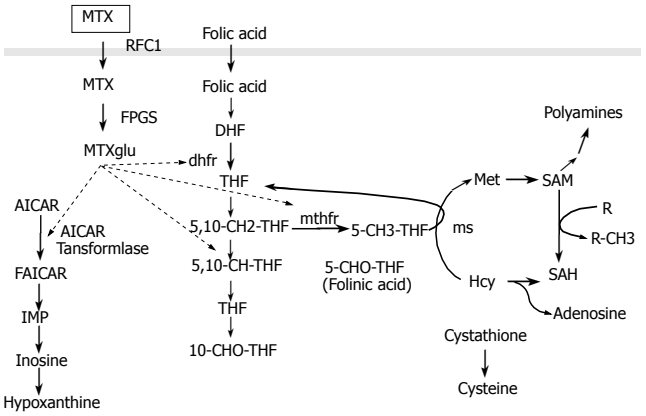


Figure 2 The dotted arrows indicate inhibitory effects of MTX methotrexate. RFC1 reduced folate carrier 1; MTXglu methotrexate polyglutamate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; DHF, dihydrofolate; THF, tetrahydrofolate; dhfr, dihydrofolate reductase; mthfr, methylene tetrahydrofolate reductase; 5,10-CH-THF, 5,10 methenyl THF; 5,10-CH2 THF, 5,10 methylene THF; 10-CHO THF, 10-formyl THF; Hcy, homocysteine; 5-CHO THF, 5-formyl THF; ms, methionine synthetase; Met, methionine; SAM, S-adenosyl-L-methionine; R, methyl acceptor; SAH, S-adenosyl–L-homocysteine.

VARIABILITY IN RESPONSE TO TREATMENT

Investigating genetic variants that influence the response to a specific drug is more difficult than finding factors that influence toxicity. Side effects are usually easy to define and identify. Efficacy scores are often less well defined, different scores to assess disease activity are used and scores [e.g. Crohn's disease activity index (CDAI), endoscopic scores, ulcerative colitis disease activity index] are usually constructed using a combination of clinical, laboratory (e.g. CRP, hemoglobin) and (or) endoscopic parameters[83]. Moreover, treatment response in a heterogenic disease like IBD is influenced by many confounding factors such as the duration of disease, disease behavior and disease severity. Besides polymorphisms in specific drug metabolizing enzymes or target proteins, heterogeneity in the genetic background can also influence response to treatment. Pharmacogenetic studies focusing on drug efficacy in IBD have been published for CSs and infliximab.

Glucocorticosteroids

CSs are effective in the initial treatment of most cases of moderate to severe active UC and CD. However, a retrospective population-based American study reported 28% and 22% steroid dependency in CD and UC patients and 16% steroid resistance after one year of treatment[84]. Other studies report steroid resistance in 20% to 40% of Caucasian IBD patients. A high frequency of surgical interventions is described in steroid dependent and resistant patients[84-86]. The doses of CSs, the tapering regimens and the definition of steroid refractoriness vary greatly between different publications. CSs are potent inhibitors of T cell activation and cytokine secretion and mediate their anti-inflammatory effect through binding the intracellular glucocorticoid receptor (GRα). A homodimer of 2 activated GRs is translocated to the nucleus and the complex subsequently binds to specific DNA sequences [glucocorticoid response elements (GRE)] and controls the expression of target genes [e.g. inhibition of the activator protein-1 (AP-1) gene and induction of the inhibitor kappa Bα (IκBα) gene](Figure 3). There are 3 potential mechanisms for resistance to CSs treatment in IBD. First, a decreased plasma level through over-expression of the MDR1 gene and subsequent elevated p-glycoprotein mediated efflux of the drug. Furthermore, an altered function of the GR and finally the anti-inflammatory capacity of CSs can be overwhelmed by an excessive synthesis of pro-inflammatory cytokines induced by the activation of pro-inflammatory transcription factors which may also reduce the affinity of GR for its intracellular ligands[87]. The reported refractoriness to CSs in IBD patients can not only be explained by disease severity since resistance to treatment is also seen in some mild cases[87,88]. Familial abnormalities of the GR have been described but are rare and there are no cases in the literature in IBD patients[89]. Steroid resistant asthma patients do not respond to high doses of inhaled CSs but they do develop the Cushingoid side effects of steroid treatment. In these patients reduced peripheral T-lymphocyte GR binding affinity and abnormalities of GR-AP-1 interaction and increased expression of GRβ (a truncated splice variant of the normal isoform GRα) are observed. GRβ is unable to activate CSs responsive genes. Honda *et al* reported GRβ specific messenger RNA expression in 83% of the patients with steroid resistant UC compared to only 9% in steroid responsive patients and 10% in healthy controls and chronic active CD patients[90]. The level of GRβ is, however only 0.165% of the level of GRα and has to be at least 5 to 10 fold in excess of GRα to significantly inhibit CSs mediated gene expression. Furthermore, *in vitro* data suggest that splicing and generation of the isoform of GR is induced by cytokines and CSs administration[90,91]. The MDR1 gene encodes the drug efflux pump P-glycoprotein-170 (Pgp-170) and is expressed on the surface of lymphocytes and intestinal epithelial cells. Pgp and MDR expression is significantly higher in CD and UC patients requiring surgery due to failure of medical therapy[92]. Genetic factors are suggested to play a role in the observed higher MDR expression. The MDR1 exonic SNPs C3435T and G2677T have been correlated

with activity and expression of Pgp170[92,93]. Although an association between CS refractory CD and MDR polymorphisms has been described, no randomized trials with fixed clinical endpoints studying the relation between these SNPs and response to steroids in IBD patients have been published[94]. Hirano *et al* found a relation between CSs administration and MDR expression and suggested that over-expression of MDR1 mRNA in peripheral blood monocytes of IBD patients and subsequent refractoriness to CSs therapy is not intrinsic but secondary to high-dose administration of CSs[95]. MDR1 knockout mice spontaneously develop colitis and the MDR1 gene maps to the IBD susceptibility locus on chromosome 7 making it an excellent functional and positional candidate gene for susceptibility to IBD. Recently associations between C3435T and UC and G2677C/T and IBD have been described[96,97]. The MDR1 3435 TT genotype was especially associated with extensive UC[97], An association between steroid refractoriness and the 3435 TT genotype can αtherefore be the result of disease heterogeneity and more specifically an extensive disease phenotype in UC. But the TT genotype is associated with lower expression of MDR1 and Pgp-170. Trials in large patient cohorts treated with fixed CSs doses and followed with well defined activity scores to assess response are necessary to further clarify the relation between MDR polymorphisms and Pgp expression and response to treatment with CSs.

Infliximab

Infliximab is a chimeric monoclonal IgG1 antibody against TNF-α. It is effective for the induction and maintenance of remission of refractory luminal and fistulizing CD. It was the first drug that clearly showed to induce mucosal healing[98-100]. Twenty-five percent of the patients however, did not respond to infliximab administration and in another 20%-30% the response was incomplete. Pharmacogenetic studies of response to infliximab in CD are interesting since most patients have been included in randomized placebo controlled phase Ⅲ and Ⅳ multicenter clinical trials. Most patients received the same dose (5 mg/kg) of the drug and response and remission were defined as a decrease of CDAI of 70 (or 100) points and drop below 150 respectively. DNA was collected from subcategories, by individual centers. Therefore the studies by different centers are well comparable. Infliximab reaches its effect in CD not only by blocking soluble TNF-α but also by inducing apoptosis of activated T-cells and monocytes[101-103]. The CARD15 gene on chromosome 16 was the first susceptibility gene identified for CD. In most Western populations 40% of CD patients carry at least one variant in the gene[104, 105]. The 3 CD associated CARD15 mutations alter NF-κB activation and subsequent cytokine production[106-108]. Since NF-κB signaling and TNF-α levels after stimulation are lower in cells carrying a CD associated CARD15 variant, it was hypothesized that patients carrying a mutation in the CARD15 gene might respond differently to treatment with a TNF blocking agent. Therefore CD patients receiving infliximab were genotyped for R702W, G908R and 1007InsC in CARD15, however no difference was observed in response measured by CDAI between patients with and without variants in

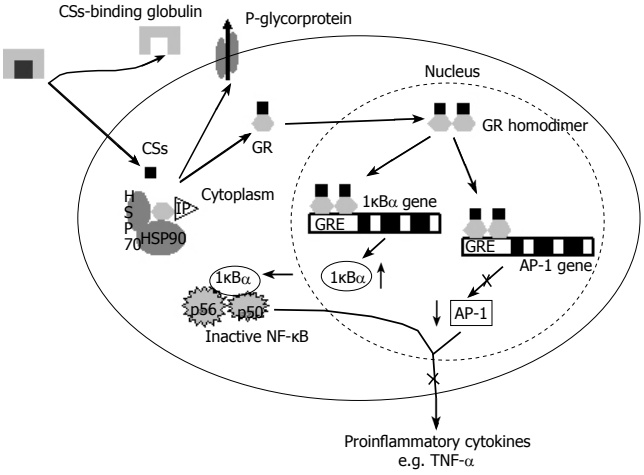


Figure 3 Anti-inflammatory mechanism of glucocorticoids (CSs). CSs enter the cell and interact with the glucocorticoid receptor (GRα) to change the GR conformation, induce formation of GR homodimers and translocation to the nucleus. GR homodimers specifically bind to glucocorticoid response elements (GRE) in target genes. HSP90, 90-kDa heat shock protein; HSP70, 70-kDa heat shock protein; IP, 56-kDa immunophilin; IκBα inhibitor kappa B alpha; NF-κB, nuclear factor kappa B; AP-1, activator protein 1.

the gene[109,110]. A possible association between SNPs in the main target molecule of infliximab TNF-α (-238, -308, -376, -857, -1031) and its receptors TNF receptor 1 (TNFR1) (-609 and +36 or P12P) and TNFR2 (168 or L56L, 587 M196A, 1663, 1690) and response to treatment in CD was studied by several investigators but no significant associations were found[111-114]. Taylor *et al* found a weak association between a haplotype spanning the TNF-α and TNF-β locus on chromosome 6p21.3 and lack of response to infliximab treatment in 75 CD patients treated with the drug. None of the individual SNPs was however associated with response to therapy[113]. Since the efficacy of infliximab in CD is partly the result of its ability to induce apoptosis of activated T lymphocytes, Hlavaty *et al* studied the effect of polymorphisms in apoptosis genes and short term clinical response in a cohort of 287 patients treated with infliximab for luminal or fistulizing CD. In luminal CD, patients carrying the Fas ligand -843 CC/CT genotypes responded in 74.7% compared to a response rate of 38.1% in patients with the TT genotype ($P < 0.01$, OR = 0.11, 95% CI 0.08-0.56). The bad outcome to infliximab in patients carrying FasL-843T could be overcome by concomitant use of AZA. Patients with the Caspase 9 93 TT genotype all responded, in contrast with 66.7% of patients with the CC and CT genotypes ($P = 0.04$, OR = 1.50, 95% CI 1.34-1.68)[115]. Binding of infliximab to membrane bound TNF-α not only induces apoptosis of monocytes and lymphocytes but probably also cell lysis by complement dependent and antibody dependent cell-mediated cytotoxicity (ADCC). The FCGR3A gene encodes FcγRⅢa, a receptor for the Fc fragment of immunoglobulines (Ig) on macrophages and natural killer cells. The non-synonymous G4985T (amino acid position Ⅴ158F) variant in the FCGR3A gene results in a different binding affinity. The FcγⅢa receptor of cells with the 158Ⅴ Ⅴ genotype have a higher affinity for the IgG Fc fragment and natural killer cells and the lysis of lymphomatous cells was more effective in patients

with this genotype treated with the TNF-α blocking agent rituximab[116,117]. Louis *et al* studied the association between this polymorphism and short term response to infliximab in 200 patients with refractory luminal or fistulizing CD. The clinical response (CDAI) was 82.9% in patients with VV genotype and 72.7% in patients with VF or FF genotypes. However, all patients with the V V genotype had a biological response (defined as a drop of CRP of at least 25% compared to baseline) compared to only 69.8% of the patients with one or more F alleles ($P < 0.01$)[118]. This study therefore suggests that there might be a better response to treatment with monoclonal antibodies against TNF-α in patient with the V V genotype.

DISCUSSION

Since the start of pharmacogenetic research 50 years ago and the identification of slow and rapid acetylators due to mutations in the NAT genes, major advances have been made. The most relevant discovery for IBD is the identification of variants in the TPMT gene and its importance for patients treated with AZA or 6-MP. To date, this is also the only test that has proven to be cost effective in clinical practice in IBD[55]. Pharmacogenetic studies of infliximab, MDR and the efficacy of CSs have contributed to understanding the mechanism of action of these drugs. There are however many problems with pharmacogenetic studies besides the normal shortcomings of association studies (i.e. low power, population stratification and multiple testing). First, pharmacogenetic data available from other diseases are not always applicable to IBD. For instance, For instance, most studies with MTX are performed in oncology patients who receive high doses of the drug during a short episode. CSs response is most extensively studied in asthma patients who are treated with inhalation therapy. Second IBD is a heterogenic disease and there are many differences regarding disease severity, location, behavior and extra-intestinal manifestations that may influence therapeutic outcome. Side effects are often rare, for instance, leucopenia and myelosuppression during AZA therapy only occurs in 1%-5% of the patients treated. This results in publications with low numbers of patients which are therefore underpowered. Multicenter studies can overcome this problem. Furthermore, exact definitions for response to therapy are necessary to replicate results in independent populations. In studies with older medications especially CSs, different doses and different tapering regimes are used. This makes the literature unorganized and comparison and meta-analyses impossible. In more recent publications however, especially those regarding biologicals and immuno suppressives, doses are calculated per kilogram body weight. Interestingly pharmacogenetic studies, in infliximab and AZA or 6-MP use strict definitions regarding therapy response. A problem especially important in patients with active IBD and an inflamed bowel is the variability of absorption of orally administered drugs. It is probably better to report drug or metabolite levels instead of the administered dose to make results comparable (e.g. TGN levels in AZA therapy). Finally, most studies investigating differences in drug response as a result of genetic polymorphisms lack explicit statements of how to translate the information for use in clinical practice. Straight clinical recommendations on how to individualize therapy based on pharmacogenetic test are essential, however the study design of most pharmacogenetic studies is inadequate to draw these sorts of conclusions. The study design of most pharmacogenetic studies is inadequate to draw these sorts of conclusions. The cost effectiveness of genotyping patients before the start of treatment should also be evaluated. We can therefore conclude that pharmacogenetics remains a promising field that already contributed to better understanding the molecular mechanisms of some of the drugs used in IBD. Until now however, the only clinical useful discovery is the relation between TPMT polymorphisms and hematological toxicity associated with thiopurine treatment. In the future it is necessary to organize studies in well characterized patients who have been uniformly treated and systematically evaluated in order to quantitate drug response and side effects more objectively. Also the prize of genotyping should be compared with the money saved by the prevention of side effects or treatment of non-responders. An effort should be made to collect genomic DNA from all patients enrolled in clinical drug trials after informed consent for future pharmacogenetic studies.

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TOPIC HIGHLIGHT

Vito Annese, MD, *Series Editor*

Family and twin studies in inflammatory bowel disease

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Abstract

Studies examining the inheritance of inflammatory bowel disease (IBD) within different family groups have been the basis for recent molecular advances in the genetics of IBD. The derived heritability in Crohn's disease (CD) is higher than in many other complex diseases. The risk of IBD is highest in first-degree relatives of a CD proband, but first-degree relatives of a proband suffering from ulcerative colitis (UC) and more distant relatives are also at increased risk. Disease concordance rates in IBD have been examined in multiplex families and in three large European twin studies.

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Key words: Crohn's disease; Family studies; Genetics; Twin studies; Ulcerative colitis

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INTRODUCTION

Attempts to increase understanding of genetics of inflammatory bowel disease (IBD) have greatly increased in frequency and reached a molecular level during the last decade. In fact, the initial interest to commence with molecular genetic studies was based on the data derived from epidemiological studies showing convincingly and consistently that the prevalence of IBD is increased among relatives with Crohn's disease (CD) and ulcerative colitis (UC). Quantitatively, the degree of familial clustering of disease may be expressed as the ratio of the risk of siblings to the reported population prevalence (λs ratio). Using this formulation, the genetic risk ratio for IBD is in the range of 15-42 for CD[1–5] and 7-17 for UC[4,5]. These λs values, especially for CD, are in the range or even higher than those reported for many other complex disorders, including type 1 diabetes (λs 15), type 2 diabetes (λs < 10), schizophrenia (λs < 10) and celiac disease (λs 7-30). Collectively, these data support a strong contribution of genes in the pathogenesis of CD in particular, although they do not provide information on the exact mode of inheritance.

TWIN STUDIES IN IBD

Although few in number, twin studies have constituted an important tool to identify the relative contribution of inherited and environmental factors in the aetiology of CD and UC. Should a disease be entirely due to genes, then its concordance in identical (monozygotic) twins would approach 100% and that in non-identical (dizygotic) twins 50%. If, on the other hand, the disease is fully dependent on extrinsic and acquired factors, then its concordance would be similar in both types of twins. In large European studies conducted in Sweden[6,7], Denmark[8] and UK[9], the concordance rate for CD in monozygotic twins was estimated at between 20% and 50%, whereas the concordance rate in dizygotic twins brought up in the same environment is less than 10 % (Table 1). More recently closer examination of the phenotypic characteristics of the Swedish cohort provided some evidence that in CD even the disease phenotype may be genetically determined[7]. Thus, in the monozygotic CD concordant pairs 7/9 were concordant for disease location, as determined by Vienna classification, and 6/9 were diagnosed within 2 years of each other. There was no significant concordance for disease behaviour, *i.e.* fistulizing or stricturing type, or extent of the disease.

The evidence from twin studies in ulcerative colitis also implies an important heritable component, although a weaker one than in CD. The concordance rate of UC in monozygotic twins was reportedly about 16% and that in dizygotic twins about 4% (Table 1) , i.e. figures markedly lower that the corresponding estimates in CD[6-9]. Along

Table 1 Concordance rates for CD and UC according to three large twin studies

	Monozygotic twins		Dizygotic twins	
	CD	UC	CD	UC
Halfvarsson[7]	50% (n = 18)	19% (n = 16)	4% (n = 26)	0% (n = 20)
Orholm[8]	50% (n = 10)	14% (n = 21)	0% (n = 27)	7% (n = 44)
Thompson[9]	20% (n = 25)	16% (n = 38)	7% (n = 46)	3% (n = 34)

the same line, concordance for phenotypic characteristics in twin pairs with ulcerative colitis is less consistent compared with CD.

In comparison to the general population, an IBD co-twin is also at increased risk of developing an IBD phenotype opposite to his/her twin partner[8]. The role of smoking habits to influence the risk and phenotype in IBD has also been confirmed by twin studies[6,8]. Orholm et al. reported that smoking was positively, although non-significantly, associated with CD and negatively and significantly with UC. In that study discordant smoking habit explained at least part of the disease discordance[8].

In summary, identical twins are significantly more likely to be concordant for IBD than non-identical twins. However, the concordance for homozygous twins does not reach a higher extent than 50% underscoring the important role of environmental causes in the pathogenesis of IBD.

FAMILY STUDIES IN IBD

Familial clustering of IBD has been documented in numerous studies. The rate at which patients with CD report a family history of CD varies from 2% to 14% and of any type of IBD from 5% to 16%, whereas patients with UC possess a family history of UC from 7% to 11% and any type of IBD from 8% to 14% (Table 2)[1,2,5,10-15].

However, it should be emphasized that the methodologies used by different family studies vary, and comparing the data between studies is not straightforward. For instance, published estimates may be biased as studies carried out in teaching hospital populations tend to overrepresent IBD patients with positive family history and more severe disease course compared to population-based studies. In addition, studies do not report results in a similar manner. Thus, some of the studies report risk for all relatives while some report risks to first-degree relatives only; in addition, the degree of risk may not be assigned to all phenotypes (UC, CD and/or IBD) in all studies. Furthermore, studies with longer follow-up time are more likely to include more family members with IBD. Therefore, age-adjusted figures when available give more reliable estimates of the true risk developing IBD. Despite of different methodologies used, in all studies the greatest risk is constantly seen in first-degree relatives, especially siblings, and a positive family history is more common in CD patients than in UC patients.

Risk of IBD in first-degree relatives

Strömgren's methodology has been most frequently used for assessment of age-adjusted relative risk. The calculations are based on the assumption that all patients live to age 70 years. Based on different studies in European and North-American white non-Jewish populations[5,13,15], the approximated age-adjusted life-time risk of developing IBD for first-degree relatives of a CD proband is 5 % and that of an UC proband 1.6%. The corresponding figures for Jewish patients are 8 % and 5.2%, respectively[15]. Family studies have repeatedly shown that the siblings of a proband are at highest risk of developing IBD, while parents have the lowest risk[13,15].

Accurate risk estimates for the offspring of CD and UC probands are difficult to calculate because many of them may not have reached the age at which the first symptoms of the disease should appear. One approach to overcome these difficulties is to calculate the prevalence proportion ratio which is estimated by dividing the observed number of offspring with IBD with the expected number of cases from the general population. In a Danish population-based study, the prevalence proportion ratios of CD and UC among offspring of patients with CD were 12.8 and 4.0, respectively, while the corresponding figures for patients with UC were 2.6 and 5.1, respectively[16]. If both parents have IBD, the risk of children to develop IBD is relatively high. Accordingly, under these circumstances about one third of the offspring have developed IBD before 30 years[17,18]. Estimations for the relative risk (relative to the population prevalence) of a sibling to develop the same disease as the proband have varied greatly. The λs ratio to a sibling to develop same disease has been estimated to be 15-42 for CD and 7-17 for UC[1-5]. Tobacco smoking probably is a strong environmental factor influencing the acquisition of the disease type. Sib pairs discordant for both smoking and IBD subtype almost always show CD in the smoking and UC in the non-smoking mate[19,20].

INFLUENCE OF INHERITANCE ON THE CLINICAL PHENOTYPE IN CROHN'S DISEASE AND ULCERATIVE COLITIS

The fundamental difficulty in exact comparison of the phenotypes in family studies lies in the fact that there is no agreed standard definition for a phenotype in either CD or UC. In fact, almost all family studies have been performed before Vienna classification of CD was adopted.

Crohn's disease

The age at onset of disease, location of disease, disease behaviour, the extent of extraintestinal manifestations and need for surgery are the most often studied phenotypic characteristics of CD. In these studies, familial aggregation and a high degree disease concordance have been confirmed, and the age at diagnosis and initial disease location were especially strongly related within generations of given families[13,15,21,22]. A high degree of concordance for disease location in parent-child and sibling pairs has also been demonstrated[13,14,22-24]. In contrast, most studies found no effect of a positive family history on severity and course of CD. There are no strong arguments for phenotypic differences between familial and sporadic

Table 2 Recent studies on the occurrence (in percent) of affected first-degree relatives in a proband with CD and UC

	Proband with CD		Proband with UC	
	First-degree relatives with CD	First-degree relatives with any form of IBD	First-degree relatives with UC	First-degree relatives with any form of IBD
Freeman (2002)[10]	8.7% (n = 1000)	-	-	-
Halme (2002)[11]	10.9% (n = 257)	15.6% (n = 257)	11.3% (n = 436)	13.8% (n = 436)
Carbonnel (1999)[12]	7.5% (n = 1316)	8.4% (n = 1316)	-	-
Peeters (1996)[13]	13.6% (n = 640)	14.5% (n = 640)	-	-
Bayless (1996)[14]	12.2 % (n = 554)	-	-	-
Satsangi (1994)[1]	-	11.5% (n = 433)	-	-
Probert (1993)[2]	9.4% (n = 424)	10.4% (n = 424)	7.1% (n = 469)	8.6% (n = 469)
Yang (1993)[15]	7.4% (n = 258)	14.0% (n = 258)	7.1% (n = 269)	8.6% (n = 269)
Orholm (1991)[5]	2.2% (n = 133)	5.2% (n = 133)	7.5% (n = 504)	8.1% (n = 504)

forms of IBD except for the age at onset[12,13,20,22,24]. High concordance rates of extraintestinal manifestations in CD have been shown in two studies[21,23] between affected first degree relatives, although extraintestinal manifestations were not associated more often in a familial disease type than in a sporadic one[25].

Ulcerative colitis

A high concordance rate for the presence of extraintestinal manifestations and for the extent of colon involvement has been reported in UC patients[23]. In a recent report the concordance rate for extension of the colon involvement was observed in 33% of relative pairs, in 47% of pairs for requirement of steroids and in 34% of pairs for a high relapse rate[21]. However, in UC families the concordance rates are less consistent than in CD families.

Genetic anticipation in IBD: fact or fallacy?

The term genetic anticipation is used when the disease severity increases and age at onset of an inherited disease decreases in subsequent generations. In IBD anticipation has been proposed to explain the observation that affected children are diagnosed an average of 12 to 23 years younger than their affected parents[21,23,26-28]. Anticipation was reported to be more marked in father-child pairs than in mother-child pairs[29].

On the other hand, some reports have provided strong evidence that anticipation does not occur in IBD and is merely caused by failure to make statistical corrections[27,30,31]. Thus, supporters of this assumption believe that a more rigorous screening or closer observation of the children of an affected parent simply result in earlier diagnosis in the offspring. Studies on genetic anticipation may also suffer from follow-up bias which implies situations where children, who are unaffected by IBD at the time of a cross-sectional survey, later develop disease.

CARD15 gene variants in familially occurring and sporadic forms of IBD

Three variants of the CARD15 gene are strongly associated with susceptibility to CD[32,33], although significant geographic and racial variations in their frequency have been described. In most Caucasian populations, at least one risk allele of the CARD15 gene is found in 44%-50% of CD patients[34,35]. In these populations the proportion of CD patients with or without documented CARD15 mutations were similar among familial and sporadic cases. On the other hand, a lower frequency (15.5%) of the risk alleles of the CARD15 gene has been described among the Finns, but in this population the1007fs allele frequency was higher among the familial (10.9%) than in sporadic CD cases (3.5%)[36]. Moreover, there is evidence for genetic heterogeneity also in other North European countries. Despite low prevalence of risk alleles of the CARD15 gene mutation the prevalence of CD and familially occurring IBD are similar as in countries of high frequency of CARD 15 risk alleles[37,38].

Familial clustering of variations in intestinal permeability

A genetically impaired intestinal barrier function has long been suspected to be a predisposing factor for CD. An increased intestinal permeability is found in nearly one fourth of the asymptomatic first-degree relatives, although similar figures have also been demonstrated in spouses of IBD patients, making it difficult to judge whether these alterations are due to genetic or environmental factors, or both[39,40]. Buhner et al. have recently shown that in healthy first degree relatives of CD patients a high mucosal permeability is associated with the presence of a CARD15 3020insC mutation, indicating that genetic factors may be involved in the impairment of the intestinal barrier function in families with IBD[41]. Recently, Stoll *et al*[42] described a significant linkage and association of CD with specific haplotypes of the DLG5 gene, coding for a membrane associated guanylate kinase possibly associated with maintenance of epithelial integrity. These studies also suggested an interaction between allelic variants of the CARD15 and DLG5 genes as risk determinants of CD[42]. It will be important to identify additional gene loci potentially interacting with the risk alleles of the CARD15 genes to modify the phenotypic features, including the intestinal permeability, in IBD.

CONCLUSION

The single strongest risk factor for developing IBD is having an affected relative with IBD. The highest risk

(> 30%) is observed in offspring of two affected parents. The age-adjusted risk of IBD is about 5% for siblings and 10% for offspring. A positive family history is more common in a CD proband than in a UC proband. Relatives of patients with CD have a higher risk of developing IBD than those of patients with UC. The rates for concordance among monozygotic twins are higher for CD than for UC. Collectively, all these family data suggest a stronger genetic influence for CD than UC. Studies showing that smoking shifts the predisposition from UC to CD indicate that not the genes alone but also environmental factors have an important role in disease expression.

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TOPIC HIGHLIGHT

Vito Annese, MD, *Series Editor*

NOD2: Ethnic and geographic differences

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Abstract

Investigations into the inheritance of the three risk alleles R702W, G908R and 1007fsInsC in NOD2 associated with susceptibility to Crohn's disease have demonstrated a remarkable amount of heterogeneity across ethnicities and populations, with regional variation across Europe for example, suggesting local founder effects. In non-Caucasian populations Crohn's disease continues to increase in incidence but this increase appears not to be a consequence of variation in NOD2, further advancing the accumulating evidence for other susceptibility loci. Frequencies of the known alleles are compared across populations in health and disease and evidence for additional alleles in NOD2 is reviewed. Based on its position on chromosome 16 coincident with some other autoimmune disease susceptibility localizations, research has targeted NOD2 variation as the potential cause of other autoimmune disorders. While these investigations have mostly returned negative findings, two diseases, Blau Syndrome and Graft versus Host Disease, have been shown to be caused by risk alleles in NOD2. As is frequent in complex disease investigations, some results await validation, but the identification of NOD2 and the differences within and across population raises intriguing questions about the population genetics of the variation at this locus.

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Key words: NOD2; Crohn's disease; Inflammatory bowel disease; Ethnicities

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NOD2 AND CROHN'S DISEASE

NOD2 (also known as CARD15) was the first gene underlying susceptibility to Crohn's Disease (CD) to be identified with three common mutations being described[1,2] and an analysis of the haplotypes carrying the three major mutations suggests that these represent three independent mutation events[3]. Two of the mutations lead to amino acid substitutions (R702W) and (G908R), while the third (L1007fs) is a single base insertion that results in a stop codon that truncates the protein. In addition, a growing number of "private" mutations that are unique to individual pedigrees have been described but supporting evidence for causality based on functional evidence is rarely reported for these rare alleles[4,5]. The majority of mutations in CARD15 that are associated with CD occur in the Leucine Rich Repeat domain-the part of the protein believed to be involved in intracellular sensing of bacterial components[4].

Each of these mutations is more properly called a "risk allele" (RA) because possessing one or more of the alleles confers an increased risk on an individual, but does not mean that disease will necessarily develop. The argument about whether the three risk alleles confer loss or gain of function remains unresolved but it is clear that the normal function of NOD2 is as a receptor for bacterially derived molecules known as pathogen associated molecular pattern (PAMP) molecules in the intestine. Our understanding of the disease processes and the link between innate and adaptive immune responses has advanced remarkably because of the identification of NOD2 in up to 50% of CD. Nevertheless carriage of risk alleles is neither necessary nor sufficient for disease.

One prediction that comes from our understanding of complex disease is that such risk alleles will occur in the healthy population albeit at lower frequencies than in disease. Allele frequencies of the three RAs in CD have been reported for many populations and are summarized in Table 1. As predicted, there is a significant elevation of the allele frequencies in CD in comparison to that in healthy controls in most populations. Since few studies report on additional mutational assessment of NOD2 in their populations, it is not possible to assess whether unique or population specific mutations exist. In a number of cases a stronger association has been reported in pediatric cohorts[5]. This result has been borne out by multivariate analysis of CD that indicates that early age of onset is significantly associated with carriage of NOD2

Table 1 Allele frequencies for the NOD2 RAs in different ethnic groups

Ethnicity /population	Frequency (patient/control) R702W	Frequency (patient/control) G908R	Frequency (patient/control) L1007fs	Other features	Reference
Greek	0.1 (0.01)	0.14 (0.03)	0.18 (0.06)	EAO	[22]
Hungary	0.16 (0.08) over all 3 mutations				[23]
Israeli Arabs	0.04 (0.01) over all 3 mutations				[12]
Israeli Jewish	0.05	0.18	0.08	pediatric	[24]
Israeli Jewish	0.06	0.11	0.06	adult	[24]
USA Jewish	0.03 (0.05)	0.09 (0.03)	0.07 (0.02)		[6]
USA Caucasian	0.06 (0.05)	0.06 (0.01)	0.13 (0.02)	EAO	[5]
USA Hispanic	0 (0.03)	0 (0.01)	0.02 (0.004)	EAO	[5]
USA African American	0.08 (0.02)	0.0 (0)	0.008 (0.04)	EAO	[5]
Australia	0.11 (0.05)	0.02 (0.10)	0.07 (0.01)		[25]
New Zealand	0.03 (0.02)	0.07 (0.05)	0.08 (0.008)		[26]
South Africa coloured	0.06 (0.03)	ND (0.005)	0.01 (0.04)		[14]
Han Chinese	0 (0)	0 (0)	0 (0)		[13]

risk alleles. This enrichment is likely to be a consequence of the stronger genetic contribution to disease observed in many predominantly inherited diseases when cohorts with an early age of onset are studied. In fact the improvement in linkage and or association evidence observed in regions identified by genome wide scans when cohorts are conditioned on carriage of NOD2 may be a consequence of the effective selection for early age of onset rather than evidence for epistasis with NOD2.

Researchers in the International IBD Genetics Consortium have compared the frequency and impact of NOD2 RAs in disease groups from fourteen centers from Europe, North America and Australia (Cavanaugh *et al* in preparation) and have found a strong association between early age of onset and the NOD2 RAs, particularly for the L1007fs allele in those families. They have also noted that the P268S allele is also strongly associated with disease, but since functional assays do not support the rare variant in disease causation it is unlikely that this allele is itself disease associated[6]. Thus this variant is likely to be in linkage with other causative allele (s) whether in NOD2 itself or at some distance from the coding region. In the search for regulatory mutations the 5' region of NOD2 has been studied but have no disease associated alleles (Schreiber pers com). Nevertheless, the recent description of remote Conserved Non-Coding regions (CNCs)[7] that are implicated in disease gene expression suggests that variation that is quite distant from NOD2 may regulate its expression and potentially impact in disease.

GEOGRAPHIC AND ETHNIC DIFFERENCES

Several authors have commented on evidence for a North-South gradient in CD in Europe with a higher frequency of disease in northern Europe than southern Europe[8]. This hypothesis is extremely intriguing given the similarities already identified between multiple sclerosis and inflammatory bowel disease. Nevertheless, the frequencies of NOD2 risk alleles do not appear to underlie this gradient. Vind *et al*[9] compared Danish and Portuguese patients and controls and found no evidence to support a North - South gradient in allele frequencies. Arnott *et al*[10] have reported that NOD2 RAs are at lower frequencies in Scottish, Irish and Scandinavian CD than in England, central Europe or North America and went on to argue that these data demonstrating regional heterogeneity across Europe are suggestive of regional founder events with respect to NOD2 risk alleles. For example, allele frequencies and Population Attributable Risk (PAR) reported for NOD2 RAs in Finland are similarly low yet the incidence of disease is relatively high[11]. These data highlight the existence of other contributing susceptibility loci in CD.

Studies in disease populations from central and southern Europe confirm a consistent pattern of association between NOD2 RAs and CD (Table 1)[10]. In North America admixture probably accounts for the low frequency of NOD2 RAs in African Americans and Hispanics[5]. Bonen *et al*[6] reported a significantly higher frequency of the G908R allele in Ashkenazi jews than in non-jewish whites and that association has been conformed in other studies[12]. At the same time the frameshift mutation that is well represented in most ethnicities is at lower frequencies in Ashkenazi Jews. With the exception of Greece and Ashkenazi Jews, the G908R allele is rarely elevated in frequency disease in most disease groups (Table 1).

In a comparison of NOD2 allele frequencies in 3575 Caucasian healthy individuals from sixteen different centers from Europe, North America and Australia, Hugot et al (in preparation) described significant heterogeneity between centers with Australia and Finland having the lowest frequencies of known variants while Belgium and Canada has the highest. Those authors also note that the frequency of the RAs is highest in central and southern Europe, in good agreement with other studies.

Analyses of non-Caucasian groups has failed to identify a significant contribution from the known NOD2 RAs. In South-East Asian populations that have been studied to date, NOD2 RAs are absent from Japanese and Korean populations (reviewed in Arnott *et al* 2004) and more recently, are also absent in Han Chinese[13]. Little information is available for South American or African populations with the exception of a recent report of low

NOD2 allele frequencies in coloured South Africans of Indian descent[14]. CD in Indians is more prevalent than in Chinese or Malays and the incidence and the prevalence of inflammatory bowel diseases appears to be increasing in Asian countries though this trend is more evident for ulcerative colitis than CD[15].

The pattern that emerges from these studies both of disease groups and healthy controls suggests that CD occurs at quite variable rates across geographical regions and that in some populations where NOD2 RA carriage rates are at a high, those alleles are present in up to 50% of individuals with CD. Nevertheless the incidence of CD in populations with substantial carriage of RA eg Belgium and Canada is remarkably similar to those with lower rates, suggesting that CD occurs in those groups because of other genetic susceptibilities, raising the issue of differential genotype by environmental interaction. ie the specific agents that initiate disease process in one population may be different to those in other populations depending on the underlying genetic susceptibility. This suggests that different micro-organisms may initiate disease in different populations depending on the underlying genetic susceptibilities of that population. Such a scenario may lead to the heterogeneity, both genotypic and phenotypic, observed in inflammatory bowel disease generally. These issues will only be resolved as further research identifies other IBD susceptibility loci and functional analyses identify the microorganisms that likely underlie disease initiation.

ASSOCIATION WITH OTHER DISEASES

Interestingly, two other diseases have been associated with NOD2 mutations. The first non IBD association was with a rare disease long known to map to the pericentromeric region of chromosome 16 that has also been shown to carry mutations in NOD2 that result in the simple Mendelian inheritance of Blau syndrome[16]. Mutations that confer susceptibility to Blau syndrome occur in the nuclear binding domain (NBD) of the protein, which is believed to be independent of LRR interactions. The second association has been shown with gastrointestinal Graft versus Host disease[17]. In this case, disease course is exacerbated in the recipient if either donor or host carries any of the three risk alleles associated with CD. This increased susceptibility to rejection is probably a function of altered epithelial reactivity in the ileum leading to an impaired microbial response. Recently there has been a suggestion that NOD2 RAs may be involved in breast cancer in the individuals with first degree relatives with lung cancer[18] however this study requires validation.

OTHER AUTOIMMUNE DISEASES

In addition a number of inherited autoimmune diseases have been shown to demonstrate linkage to the pericentromeric region of chromosome 16 that houses NOD2, including rheumatoid arthritis and lupus erythematosus. As a consequence of the observation that autoimmune disease appear to cluster in specific regions of the genome[19] and might therefore share some common underlying genetic susceptibilities, a number of researchers have tested their different disease groups for the common mutations in NOD2. Diseases in which association with known NOD2 mutations have been investigated are shown in Table 2 along with evidence for linkage evidence to chromosome 16 and ethnicity of patient (and control if reported). The small subset of patients with spondyloarthropathy with chronic gut inflammation show association with NOD2 RAs and share many features with CD[20]. Those authors commented that this group of patients is likely to progress to fulminant CD. The closely related phenotypes of atopic eczema, asthma and allergic rhinoconjunctivitis[21] have been shown to have an association with NOD2 variants, although the causative alleles were not identified. With these exceptions, these studies have failed to demonstrate a fundamental association of autoimmune diseases with NOD2, nevertheless the central role that the described NOD2 RAs have in CD remains unequivocal.

Table 2 Results of association analysis between NOD2 RAs and other inherited diseases

Disease	Linkage to chromosome 16	Ethnicity	Association	Reference
Allergy and atopy	yes	German	yes[1]	[21]
Behcet's disease	yes	UK, Turkish and Arab	no[2]	[27]
Breast cancer with lung cancer FDR[3]	no	Poland	yes	[18]
Idiopathic pulmonary fibrosis	unknown	Italian	no	[28]
Necrotising enteritis	unknown	France	no[4]	[29]
Psioratic arthritis	yes	Newfoundland	yes	[30]
Psioratic arthriris	yes	UK	no	[31]
Rheumatoid arthritis	no	USA	no[5]	[32]
Sarcoidosis	no	Danes	no	[33]
Sarcoidosis	no	United Kingdom	no	[34]
Spondyloarthropathy with chronic gut inflammation	no	Belgium	yes	[20]
Systemic lupus erythematosus	yes	Spain	no	[35]
Type 1 diabetes	yes	Scandinavia	no	[36]
Wegener's granulomatosis	no	North America	no	[37]

[1]Both disease and protective associated haplotypes are described; [2]Direct sequencing to identify polymorphisms failed to identify association by case-control; [3]FDR first degree relative. The association reported occurs only in breast cancer patients who have a first degree relative with lung cancer but not in individuals without; [4]All exons and exon/intron boundaries of NOD2 were sequenced; [5]Genoyped for 3 common and a further 12 SNPs in NOD2 with no haplotype association observed.

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TOPIC HIGHLIGHT

Vito Annese, MD, *Series Editor*

OCTNs: Will the real IBD5 gene please stand up?

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Abstract

Crohn's disease and ulcerative colitis are inflammatory disorders of the gastrointestinal tract with a substantial heritable component. The IBD5 region on chromosome 5q31 is one of only two loci widely confirmed to be associated with Crohn's disease in multiple independent cohorts. Although many populations have demonstrated association with IBD5, there remains uncertainty as to the causal variant within the region. A recent report identified polymorphisms in *SLC22A4* (*OCTN1*) and *SLC22A5* (*OCTN2*) as being responsible for the IBD5 association, however, these findings have not been replicated. This review discusses the data evaluating the IBD5 locus and the OCTN genes and their relationship to inflammatory bowel disease. Several other genes, including *IRF1* and *P4HA2* may be equally as likely to contain the IBD5 causal variant as the *OCTN* genes.

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Key words: IBD5; Inflammatory bowel disease

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INTRODUCTION

The pathogenesis of inflammatory bowel disease (IBD) is believed to involve exposure to an as yet unidentified environmental trigger in a genetically susceptible individual[1]. The importance of genetic factors in the pathophysiology of IBD has been repeatedly established by the substantially increased relative risk to siblings of affected individuals and higher monozygotic versus dizygotic twin concordance rates[2-6]. While the heritability of Crohn's disease (CD) seems higher than that for ulcerative colitis (UC), the available epidemiological and genetic data suggest that there are common susceptibility factors for both diseases and it is likely that gene-gene and gene-environmental interactions determine the specific phenotype[6].

Significant progress has been made in identifying genetic factors responsible for IBD with numerous replicated susceptibility loci identified. However, among the confirmed susceptibility loci identified through linkage for IBD (IBD1-IBD6)[7-12] only the susceptibility gene within the IBD1 locus has been identified[13,14]. The caspase recruitment domain-containing protein 15 (*CARD15*) gene was described simultaneously by a positional and candidate gene approach and data from numerous populations have confirmed an association between three variants within the *CARD15* (*NOD2*) gene and CD[13-15]. Sub-phenotypic analysis shows that mutations in the *CARD15* (*NOD2*) gene are particularly associated with the development of small bowel CD[15]. Despite widespread replication of the *CARD15* (*NOD2*) findings, there is still important ethnic and geographic heterogeneity. For example, despite general genetic similarity between Ashkenazi Jews (AJ) and Non-Jewish (NJ) Caucasians, the allele frequencies and odds ratios of the *CARD15* (*NOD2*) variants for AJ are slightly different when compared to those found in NJ populations and in Asian individuals affected by CD, *CARD15* (*NOD2*) variants are extremely rare and not associated with CD[15-18].

IDENTIFICATION OF THE IBD5 LOCUS

The IBD5 locus on chromosome 5q31 is the only other locus clearly demonstrated to confer increased risk for CD through association. Following an initial finding of significant linkage in a Canadian population[12], subsequent studies identified a 250 kb risk haplotype within the IBD5 locus that is significantly associated with CD[19]. As part of this effort, the region was sequenced in eight individuals and was intensively evaluated with dense genotyping in more than 250 trios which led to the discovery of long segments of complete linkage disequilibrium (LD), described as "haplotype blocks", which were separated by apparent "hotspots" of recombination[20]. This original study described 11 haplotype tagging (single nucleotide polymorphisms) SNPs in this region which were equally associated with CD. The 250 kb region has since been the subject of intensive study as a number of highly relevant potential candidate genes are located within and nearby to it. These include interferon regulatory factor 1 (*IRF1*), solute carrier family 22, member 4 (*SLC22A4*) also known

as organic cation transporter 1 (*OCTN1*); solute carrier family 22, member 5 (*SLC22A5*), also known as organic cation transporter 2 (*OCTN2*); and prolyl 4-hyroxylase (*P4HA2*) among others and there are also a number of important cytokine genes (*IL-4, IL-13, IL-5, IL-3*) located adjacent to the immediate 250 kb risk haplotype. Since the initial identification of this risk haplotype, numerous studies have replicated the association with CD and a few have demonstrated an association between IBD5 and UC as well[21-25], establishing this as one of a still limited number of *bona fide* associations in human complex disease genetics and the second in IBD specifically.

THE IBD5 SUSCEPTIBILITY GENES?

In 2004 Peltekova and colleagues postulated that functional polymorphisms, L503F (rs1050152) and G-207C (rs2631367) in the *SLC22A4* (*OCTN1*) and *SLC22A5* (*OCTN2*) genes, respectively, comprised a two locus risk haplotype that accounted for the association findings at IBD5[26]. The "TC haplotype" described was found in 54% of CD patients versus 42% of affected controls ($P = 0.0003$). Moreover, the odds ration for those homozygous for the TC haplotype was 3.43-5.14 for CD with an even higher odds ratio for those carrying the TC haplotype and a CARD15 risk allele (7.28-10.5). No association was found with ulcerative colitis. In this study, the TC haplotype was independently associated with CD as compared to the background risk haplotype where the marker IGR2078 was utilized as a tagging SNP to represent the IBD5 risk haplotype.

That functional polymorphisms in significant linkage disequilibrium with each other in adjacent, highly homologous genes may act as a functional cassette to increase disease risk would represent a novel, important paradigm in complex disorders. In particular, *SLC22A5* (*OCTN2*) transports carnitine, which plays a key role in mitochondrial transport of long chain fatty acids; furthermore, *SLC22A5* (*OCTN2*)-deficient mice develop intestinal lymphocytic infiltration. However, establishing a functional role for SNPs located within conserved elements which subtly alter gene expression can be difficult compared to establishing altered activity for amino acid polymorphisms.

REPLICATION STUDIES AND GENOTYPE PHENOTYPE STUDIES OF THE IBD5 LOCUS

As described above, there have been numerous studies to confirm the association of CD with the IBD5 risk haplotype[21-25]. These studies initially tested varying markers selected as tagging markers for the risk haplotype on chromosome 5 due to the high degree of linkage disequilibrium in the region. Statistical support for a relatively unique contribution of the two-locus *OCTN* haplotype was initially described, but not examined in large enough cohorts to confidently refine localization in the IBD5 region[27]. Further studies performed subsequent to the identification of the OCTN variants have primarily tested these markers alone although more recent reports have also tested additional SNPs within the IBD5 risk haplotype.

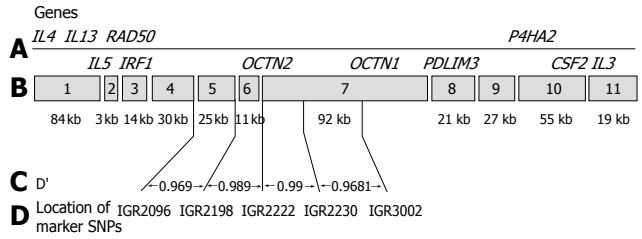


Figure 1 IBD5 map demonstrating the location of SNPs and regional candidate genes (Taken from Noble et al ref 28 - permission for reproduction being sought).

A well designed study including 374 patients with CD from Noble and colleagues in Edinburgh demonstrated that while the OCTN1 and OCTN2 variants are associated with CD, this association is not independent of the background risk haplotype in the region[28]. This study assessed not only the OCTN variants but also a number of surrounding SNPs from the list of SNPs comprising the IBD5 risk haplotype (IGR2096, IGR 2198 and IGR2230). Utilizing these SNPs they could not demonstrate independent association of the OCTN variants and suggest that the IGR2078 SNP used by Peltekova and colleagues is one haplotype block removed from the OCTN variants and therefore was not truly indicative of an independent association (Figure 1). A number of additional such reports have been presented in abstract form with similar findings (DDW 2005).

Despite the controversy that still exists over the true causal gene within the IBD5 locus, studies performed both before and after the identification of the OCTN variants have attempted to further dissect the specific phenotype associated with this region. Just as is the case for the causal genetic variant there is also inconsistent data regarding the phenotype associated. Early on, it was reported that the IBD5 locus was associated with perianal CD in a UK study[21]. This report analyzed several of the SNPs on the IBD5 risk haplotype in a case-control fashion. More recently, association of the IBD5 region has been found to be associated with markers of more severe disease such as progression to stricturing or penetrating CD and need for surgery in an Edinburgh population[28]. No association was found for any specific disease location or with UC in this report. A study from the same investigators in a pediatric population also confirms the association of the OCTN variants with CD and UC but the variants were not independently associated from the surrounding SNPs of the IBD5 risk haplotype[29]. This study also demonstrated that the OCTN TC haplotype may be a marker for more severe CD with an association with lower height, weight and body mass index (BMI) at the time of diagnosis. A report evaluating IBD patients from Italy also demonstrated IBD5 to be related to more severe CD behaviour although by multiple regression it was felt that this effect was essentially conferred by the presence of CARD15 risk alleles rather than by IBD5 loci independently[30].

A study of more than 981 IBD patients from Leuven

could not demonstrate an association of the OCTN1 and OCTN2 variants with IBD, CD or UC globally but did show an association with perianal and penetrating CD[31]. These association findings have yet to be replicated and their significance has yet to be determined. One may speculate that the IBD5 locus represents a "phenotype-determining" genetic effect, however, the lack of an overall positive association with CD in the Belgian population makes this interpretation somewhat precarious. There have been inconsistent reports describing a role of IBD5 and UC with only a few describing positive findings[22,23,32]. A study of 187 German families with UC was the first to describe an association of IBD5 with UC by family-based association testing and, in particular, the association was strongest in those individuals carrying one of the CARD15 risk alleles[22]. This potential epistatic interaction was also reported in a cohort from the UK[23]. Although these results require further investigation in larger sample sizes, it is not altogether unexpected that there may be some effect of IBD5 on the development of UC. There is an increased risk of UC developing in the relatives of those with CD and the reverse is also true suggesting there are genes which confer overlapping susceptibility[6]. In addition, the clinical heterogeneity among the two disorders could result in misspecification of disease type in those with colonic involvement. Moreover, it has been previously noted that there are subsets of CD which have a more "UC-like" appearance raising further suspicion for overlapping genetic susceptibility factors[33]. One might also speculate on the possibility of a unique "IBD5 positive", genetically and clinically unique subset of IBD patients, that would require further testing to be better characterized. As is the case with CARD15, the IBD5 region does show some variation with respect to the population studied. Studies from Asian populations show no evidence for IBD5 as a contributing factor to IBD susceptibility[34,35]. In addition, a study with a significant Jewish population report no evidence for a contribution of IBD5 to susceptibility in this high risk group[36].

In conclusions, while common alleles of modest effect play a very important role in the architecture of complex diseases and can be readily detected in some cases with modest sample sizes, it is now widely recognized that extremely large sample sizes are required in order to conclusively confirm these factors, resolve their location through fine-mapping and identify specific subphenotypes that are either preferentially associated or not-associated with disease. While the two allele risk haplotype of the *SLC22A4* (*OCTN1*) and *SLC22A5* (*OCTN2*) genes may thus far be the best candidates for the IBD5 association findings, there is as yet insufficient evidence to make concrete conclusions regarding their significance. Current data suggests that other, nearby SNPs, can be considered statistically equivalent in terms of their association to CD and the extensive linkage disequilibrium in this region suggests that caution must be exerted in asserting which of the SNPs in this region is potentially causal. Further functional evaluation of these variants is certainly warranted. In addition, the inconsistent findings in UC illustrate the importance of better powered UC genetics studies to further delineate the role of IBD5 and UC susceptibility and phenotype. In complex disorders, the IBD5 association represents one of the best replicated genomic regions, and therefore warrants comprehensive studies in large, well-phenotyped cohorts to define those population subsets and functional polymorphisms which most likely contribute to disease risk. To further advance the understanding of the IBD5 locus and its role in IBD susceptibility will require even larger population studies than those already performed, that will have adequate power to dissect the high degree of linkage disequilibrium found in this region in conjunction with functional studies that will demonstrate mechanisms. Due to the modest but important genetic relative risk of this locus, large consortia with prospectively collected, well-phenotyped cohorts will be required in order to refine the information regarding this region.

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REVIEW

Non invasive fibrosis biomarkers reduce but not substitute the need for liver biopsy

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Abstract

Chronic liver diseases are very common worldwide, particularly those linked to viral hepatitis and to alcoholic and non-alcoholic fatty liver. Their natural history is variable and long-term evolution differs in individual patients. Optimised clinical management of compensated chronic liver diseases requires precise definition of the stage of liver fibrosis, the main determinant of prognosis and of most therapeutic decisions. Liver biopsy is the gold standard for assessment of hepatic fibrosis. However, it is invasive with possible complications, costly and prone to sampling errors. Many non-invasive markers of liver fibrosis have been recently proposed and assessed in the clinical setting as surrogates of liver biopsy. Direct markers are based on biochemical parameters directly linked to fibrogenesis while indirect markers use simple or more sophisticated parameters that correlate with liver fibrosis stages. Non-invasive markers of liver fibrosis have been tested in different forms of chronic liver disease and showed variable diagnostic performance, but accuracy rarely was above 75%-80%. Better results were obtained when markers were combined. On this line, we have recently proposed a set of algorithms that combine sequentially indirect non-invasive markers of liver fibrosis, reaching 90%-95% diagnostic accuracy with significant reduction in the need for liver biopsy. Based on available evidence, it can be anticipated that non-invasive markers of liver fibrosis and their combined use will soon become a most useful tool in the clinical management of many forms of chronic liver disease. However, their implementation is expected to reduce, but not to completely eliminate, the need for liver biopsy.

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Key words: Chronic liver diseases; Hepatic fibrosis; Liver biopsy; Non invasive fibrosis markers

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INTRODUCTION

Chronic infection with the hepatitis B (HBV) and C (HCV) viruses and alcoholic and non-alcoholic steatohepatitis are the main causes of chronic and progressive liver disease leading to cirrhosis, end-stage liver disease, and hepatocellular carcinoma worldwide, with a predominant role of hepatitis B in the Middle and Far East regions and of hepatitis C and steatohepatitis in the Western Countries. These different etiologic forms of chronic liver disease (CLD) have a common histopathological pathway that is the formation and accumulation of fibrosis leading to the development of progressive distortion of the hepatic architecture that is the hallmark of evolution to cirrhosis. Natural history studies indicate that advanced fibrosis and cirrhosis develop in about 20%-40% of patients with chronic hepatitis B or C and in a similar proportion of those with alcoholic or non alcoholic steatohepatitis[1-4]. Development of fibrosis is a step-by-step process starting from minimal fibrosis limited to the portal tracts, followed by more extensive fibrosis with septa expanding into the liver parenchyma, that can form bridges between two portal tracts or portal tracts and central veins, eventually ending in complete cirrhotic nodules. This type of progression may take years or decades to fully develop, and staging of hepatic fibrosis is therefore of paramount clinical importance for the prognostic assessment in the individual patient. In patients with chronic viral hepatitis precise definition of the hepatic fibrosis stage is the most important parameter to assess the risk of disease progression and to decide the need for immediate antiviral therapy. This is particularly true for those patients with chronic viral hepatitis or alcoholic or non-alcoholic fatty liver who are still in a well compensated phase and have no overt clinical or laboratory signs of cirrhosis. In these patients liver biopsy represents the gold standard for evaluating presence, type and stage of liver fibrosis. This procedure, however, is invasive, costly and difficult to standardise. In recent years there has been increasing interest in the possibility of identifying and describing liver fibrosis by using non invasive, surrogate markers measurable in the peripheral blood and many of such tests have been reported in the literature. This review is

aimed to describe the different non invasive markers and methods that have been proposed for the assessment of liver fibrosis, to discuss their advantages and limits and to suggest a rational use in clinical practice.

HISTOLOGICAL CLASSIFICATION OF CHRONIC LIVER DISEASE

The old classification of chronic hepatitis made a rough grading distinction between milder and more severe forms of liver disease. More recently, the new insights in the etiology and therapy of CLDs, particularly viral hepatitis, has led to a revised classification, aimed to describe and quantify in more details necroinflammation and fibrosis. Several semiquantitative scoring systems have been proposed to measure the activity grade of inflammation and to stage the amount and type of fibrosis in the liver. Scoring systems specifically designed for chronic viral hepatitis are the histological activity index proposed in 1981 by Knodell *et al*[5], the Ishak's score[6] and the METAVIR scoring system[7].

The histological activity index is based on the evaluation of four parameters: periportal necrosis (score of 1-10), parenchimal damage (score of 0-4), portal inflammation (score of 0-4) and fibrosis (score of 0-6). The cumulative score therefore ranges from 0 to 18 to describe the overall histological activity. The limitation of this scoring system is that necro-inflammation (grading) scores are cumulated with the fibrosis (staging) scores, while these parameters describe different lesions and clearly have different prognostic implications. The Ishak's system is a revised version of the histological activity index, and describes the activity grade and the fibrosis stage as two separate items. Liver fibrosis is here classified in absent (0), mild (1-2), moderate (3-4) and severe/cirrhosis (5-6). The METAVIR scoring system for liver fibrosis, frequently used in recent times particularly for chronic hepatitis C, is described in details in Table 1. All these scoring systems have some limits, being semiquantitative, not linear and prone to intra- and inter-observer variation and to sampling variability[8]. They have been more validated for clinical use with some but not all the different etiological forms of CLD that are characterised by progressive fibrosis leading to cirrhosis.

Table 1 METAVIR scoring system for liver fibrosis

Stage	Description
F0	No fibrosis
F1	Portal fibrosis without septa
F2	Portal fibrosis with few septa
F3	Septal fibrosis without cirrhosis
F4	Cirrhosis

Portal fibrosis is a stellate enlargement of portal tracts without any bridging fibrosis on the biopsy sample. Few septa mean at least one fibrous septa on the core biopsy. Theoretically, a fibrous septa is a bridge of connective tissue between two portal tracts, a portal tract and a centrolobular vein, or between two centrolobular veins. Septal fibrosis means that the liver biopsy is crossed by several septa; the transition between F2 and F3 begins when there is more fibrous septa than portal tracts without septa on the biopsy. Cirrhosis means that liver tissue is mutilated by nodular fibrosis that delineates hepatocytes nodules.

LIVER BIOPSY: PROS *VS* CONS

For many years liver biopsy has been considered the golden standard for the evaluation of liver fibrosis staging. Liver biopsy has the advantage of allowing to obtain information not only on fibrosis, but also on many useful parameters, such as inflammation, necrosis, steatosis, hepatic iron and so on. Furthermore, it allows to identify suspected or unexpected cofactors and comorbidities. However, liver biopsy has also a number of limitations that have to be taken into account. Many recent studies clearly indicate that liver biopsy, as it is usually taken for diagnostic purpose, is prone to sampling errors and may underestimate the amount of liver fibrosis. Several studies suggest that cirrhosis might be missed on a single blind percutaneous liver biopsy in 10%-30% of cases[9-11]. When three different liver samples were analysed, the percentage of correct diagnosis increased from 80 to 100%[12]. In more recent times, Regev and colleagues have shown that samples obtained from the right and left lobes of the liver during laparoscopy gave different fibrosis staging in one third of the cases[13]. Other studies have analysed agreement/disagreement among pathologists. Although the use of more standardised scoring systems, such as those of the Knodell's, Ishak's and METAVIR's classifications, has improved the inter-observer and intra-observer variability, there are still several factors that may significantly influence the diagnostic accuracy of a liver biopsy. The size of the liver sample is obviously very important. Colloredo *et al* have carefully analysed the importance of the sample size for a correct stadiation of liver disease in patients with chronic hepatitis C[14]. By reducing progressively the dimension of the same liver biopsy, they reported that the smaller was the sample analysed, the milder was the diagnosis made by the pathologist in relation to the stage of fibrosis. The same biopsy was diagnosed as F3-F4 when a larger part of the sample was observed and only as F1-F2 when the size of tissue was reduced in length or wideness. Other studies have reported that the type and the size of needle used is also important. The Tru-Cut needle was superior to the Menghini needle, particularly for the diagnosis of more advanced fibrosis[15-16]. A thick needle was superior to the fine needle in assessing the presence of advanced fibrosis and cirrhosis[17]. Some studies would suggest that an adequate liver biopsy sample should contain more than 5 portal tracts and be at least 15 mm in length[18-20]. However, other studies reported higher threshold for optimised accuracy. Colloredo *et al* concluded that an adequate specimen should be at least 20 mm in length with at least 11 complete portal tracts while others have recommended even bigger samples, up to 25 mm in length[14,21]. On the same line, Scheuer has recently concluded that bigger is better[22]. The need for obtaining a liver sample of adequate size is however in contrast with the patient need of a procedure causing limited pain and hemorrhagic risks. Liver biopsy may be in fact a risky procedure for some patients, particularly for those with more advanced liver

fibrosis[23-25]. A French survey which interviewed 1177 general practitioners concluded that liver biopsy may be refused by up to 59% of patients with chronic hepatitis C and that 22% of the physicians share the same concern for this invasive procedure[26]. On this line, a recent survey assessing the consensus among Italian hepatologists on when and how to take a liver biopsy in chronic hepatitis C showed great divergence in the management of the same subgroup of patients[27]. Most recently Rousselet *et al* reported that the degree of experience of the pathologist, as indicated by longer duration of practice or belonging to an academic setting, may have an outstanding impact on the diagnostic interpretation of liver biopsy, even higher than that determined by the related to sample size[28].

Another shortcoming of liver biopsy is its cost as it always requires hospitalisation for 6-18 hrs. A cost-benefit analysis showed that in US the cost of a liver biopsy is 1032 USD and it could raise to 2745 USD when complications occur[29].

PATHOPHYSIOLOGY OF HEPATIC FIBROSIS-THE CONCEPTUAL BASIS OF NON INVASIVE BIOMARKERS

The key step in the pathophysiology of liver fibrogenesis is the balance between extracellular matrix (ECM) deposition and removal. Indeed, the ECM metabolism is a very dynamic process, influenced by factors that contribute to its deposition and by others that mediate its degradation. The hepatic stellate cells (HSCs) are the major source of ECM. During liver injury, activation of quiescent HSCs to a proliferative, fibrogenic and contractile type of myofibroblasts is the dominant event in fibrogenesis. HSCs can be activated by several cytokines (e.g., tumor growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), platelet derived growth factor (PDGF)) which are secreted in response to liver injury. On the other hand, other signals (e.g., interleukin-10 (IL-10)) promote ECM degradation. Once activated, HSCs secrete cytokines such as metalloproteinases, TGF-β1, PDGF, monocyte chemotaxis protein 1 (MCP-1), endothelin 1 (ET-1). Some of these are directly involved in fibrogenesis (TGF-β1, connective tissue growth factor), others in chemotaxis (MCP-1) and proliferation of HSCs (PDGF, ET-1) and others in matrix degradation (metalloproteinases)[30].

The pathogenesis of liver fibrosis is somehow related to the etiology of the underlying CLD. The mechanisms by which HCV and HBV induce liver fibrosis are only partially understood. In chronic hepatitis B infection the pathogenesis of hepatic fibrosis has been associated with cytokines, particularly TGF-β1[31]. HCV induces oxidative stress and recruitment of inflammatory cells, with HSCs activation and collagen deposition. In addition, several HCV proteins directly stimulate the fibrogenic and flogistic pathways of HSCs[32]. A recent "in vitro" study reported that hepatitis C virus nonstructural genes (NS3 and NS5B) are able to induce increased expression of TGF-β1 and of other profibrogenic factors in infected hepatocytes[33]. These cellular events directly mediated by HCV in infected hepatocytes could explain the occurrence of progressive liver fibrosis with minimal inflammation. The pathogenetic mechanisms acting in alcoholic (AFLD) and in non alcoholic fatty liver disease (NAFLD) also involve cytokine- and oxidative stress- mediated injury. In AFLD the main stimulus for cytokine release from Kupffer cells in the liver is portal endotoxemia, arising as a result of increased gut permeability caused by ethanol and its metabolism to acetaldehyde. Oxidative stress derives from ethanol metabolism, Kupffer cell activation and from the effect of TNF-α on hepatocyte mitochondria. The resulting activation of HSCs increases the fibrogenic and inflammatory signals[34-35]. The pathogenesis in non alcoholic steatohepatitis (NASH) is less understood and several mechanisms have been proposed and investigated. Hepatic steatosis is considered as the first of two hits in the pathogenesis of NASH, since the presence of oxidisable fat within the liver is able to trigger lipid peroxidation[36]. However, many patients with fatty liver do not progress to necroinflammation and fibrosis. The potential second hits for the development of NASH could be an increase in the expression of ethanol-inducible cytochrome P450 2E1 (CYP2E1) and in the intrahepatic concentration of free fatty acids, resulting in oxidative stress via peroxisomal oxidation[37]. It has also been hypothesised that iron, in relatively low concentrations, could synergize with lipid overload and CYP2E1 induction to trigger oxidative stress in hepatocytes[38].

All these data and concepts are of great interest for the understanding of the various factors that contribute to progressive liver fibrosis in patients with different forms of CLD. However, their complexity and interplay clearly explain the difficulties encountered in the search of a specific and sensitive marker that could be universally valid as a diagnostic and prognostic tool to measure liver fibrogenesis in clinical practice.

GENETIC POLYMORPHISMS AND LIVER FIBROSIS

The complexity of the fibrogenetic process and the high number of cytokines involved imply that several genetic polymorphisms could influence progression of liver fibrosis. To date, genetic polymorphisms linked to hepatic fibrogenesis have been investigated mainly in chronic hepatitis C and in AFLD. Table 2 describes gene polymorphisms that were reported to either favour or reduce fibrogenesis in patients with different forms of CLD. While these studies clearly indicate that many genetic factors have a definitive influence on the risk of developing a more or less active and progressive fibrogenesis, none of them have found an application as diagnostic/prognostic marker in clinical practice, due to their complexity, difficulty to test and variable behaviour in different patients populations. Better understanding of the genetic influence on liver fibrogenesis is nevertheless of paramount importance as it may lead in the near future to identification of new therapeutic targets and strategies for development of effective antifibrotic treatments.

Table 2 Gene polymorphisms described as involved in fibrogenesis

Author	Etiology of liver disease	Gene	Function of the gene product	Implicated genotype	Effect on gene product	Effect on fibrosis
Powell[39]	HCV	TGF-β1	Profibrogenic	Codon 25 (pro/arg)	Increased transcription	Increased
Powell[39] Forrest[40]	HCV	AT	Profibrogenic	-6 G/A	Increased transcription	Discordant results
Bonkovsky[41] Negro[42] Chitturi[43] Geier[44]	HCV / NASH	HFE	Iron metabolism	C282Y, H63D	Iron overload	Discordant results
Yee[45] Grove[46]	HCV /AFLD	TNF-β	Proinflammatory	-308 G/A	Increased transcription	Increased
Grove[47] Knapp[48]	HCV / AFLD	IL-10	Immune modulation	-1082 A/A; -627 C/A	Decreased transcription	Increased
Reynolds[49]	HCV	MPO	Battericide	-463 G/A	Increased transcription	Increased
Wozniak[50]	HCV	ApoE	Viral entry to cells	E4 allele	Abnormal function	Reduced
Muhlbauer[51]	HCV	MCP-1	Proinflammatory	-2518 G/A	Increased transcription	Increased
Wright[52]	HCV	Factor Ⅴ Leiden	Thrombine generation	Codon 560 (arg/gln)	Resistance to activation	Increased
Romero-Gomez[53]	HCV	SLC11A1	Macrophage function	Homozygosity $(GT)_5AC(GT)_{10}G$ (Allele 2) in the promoter region	Poor promoter	Reduced
Adinolfi[54]	HCV	MTHFR	Folate metabolism	C677T	HyperHC	Increased
Okamoto[55]	HCV	MMP	Matrix degradation	1G/2G (MMP-1), 5A/6A (MMP-3), C/T (MMP-9)	Reduced transcription	More frequent in cirrhosis than CHC
Yamauchi[56] Okamoto[57] Frenzer[58]	AFLD	CYP2E1	Ethanol metabolism	c1/c1, c2/c2 alleles	Increased activity	Discordant results
Yamauchi[56] Frenzer[58]	AFLD	ADH	Ethanol metabolism	ADH2 c1,c2,c3 alleles; ADH3 c1, c2, c3 alleles	Abnormal function	Discordant results
Yamauchi[56] Okamoto[57]	AFLD	ALDH	Ethanol metabolism	ALDH2 c2/c2 alleles	Abnormal function	Discordant results
Burim[59]	AFLD	CYP1A1	Ethanol metabolism	m2/m2 allele	Increased activity	Increased
Dixon[60]	NAFLD	AT and TGF-β	Profibrogenic	High AT and TGF-β1 producing polymorphisms	Increased activity	Increased

TGF-β = transforming factor beta; AT = angiotensinogen; HFE = hereditary haemochromatosis gene; NASH = non alcoholic steatohepatitis; TNF-β = tumor necrosis factor beta; AFLD = alcoholic liver disease; IL-10 = interleukin 10; NAFLD = non alcoholic fatty liver disease; MPO = myeloperoxidase; ApoE = apolipoprotein E; MCP-1 = monocyte chemotactic protein 1; SLC11A1 = solute carrier family 11 member 1; MTHFR = methylenetetrahydrofolate reductase; HC = homocisteinemia; MMP = metalloproteinase; CYP2E1 = cytochrome P 2E1; ADH = alcohol dehydrogenase; ALDH = aldehyde dehydrogenase; CYPA1 = cytochrome P A1.

SEARCHING FOR THE IDEAL NON INVASIVE MARKER OF LIVER FIBROSIS: THE HOLY GRAIL OF THE CLINICAL HEPATOLOGIST

In the last decade, many studies have been dedicated to the search of non invasive markers able to provide an accurate information about liver fibrogenesis activity and fibrosis stage in patients with chronic, potentially progressive, hepatic diseases. The ideal characteristics of such a marker are summarised in Table 3. Two main, quite different, approaches have been followed. Many studies have been dedicated to the evaluation of "direct" markers of fibrogenesis, i.e. of biochemical parameters, measurable in the peripheral blood as direct expression of either the deposition or the removal of ECM in the liver. These direct markers of liver fibrosis include several glycoproteins (hyaluronan, laminin, human cartilage glycoprotein 39 (YKL-40)), the collagens family (procollagen Ⅲ, type Ⅳ collagen and type Ⅳ collagen 7s domain), the collagenases and their inhibitors (metalloproteinases and tissue inhibitors of metalloproteinases) and a number of cytokines connected with the fibrogenetic process (TGF-β1, TNF-β). These markers and their role in fibrogenesis are described in Table 4. The potential clinical applications of such markers appear extremely interesting and innovative, as they could be used not only to stage liver fibrosis, but also and more appropriately to assess the speed of liver fibrogenesis with most relevant prognostic value, and also to estimate and monitor the efficacy of and the response to antifibrotic drugs. However, these ambitious goals have not been yet achieved and the described direct makers of fibrogenesis have been so far tested only for their performance in defining the actual

Table 3 Features of the ideal marker of liver fibrosis

Specific for fibrosis of the liver
Providing measurement of: A) stage of fibrosis, B) fibrogenesis activity
Not influenced by comorbidities (e.g. renal, reticulo-endothelial)
Known half-life
Known excretion route
Sensitive
Reproducible

Table 4 Direct non invasive markers of liver fibrosis

Author	Liver disease	Marker	Characteristics	Pathophisiology
McHutchison[61] Murawaki[62] Halfon[63] Pares[64] Suzuki[65] Naveau[66] Santos[67] Montazeri[68]	HCV, AFLD, NAFLD, HBV	Hyaluronic acid	Component of the ECM in every tissue	Synthesized by HSCs and degraded by sinusoidal endothelial cells
Santos[67] Walsh[69]	HCV, NAFLD	Laminin	Co-expressed with type Ⅳ collagen in basement membranes	Increased deposition in viral disease
Tran[70] Saitou[71]	AFLD, HCV	YKL-40	Human cartilage glicoprotein	Involved in remodelling and degradation of ECM
Sakugawa[72] Murawaki[73] Walsh[69] Saitou[71] Santos[67]	HCV, NAFLD	Type Ⅳ collagen/ 7s domain	Main collagen component of the basement membrane	Increase with severity of liver fibrosis
Guechot[74] Pares[64]	HCV, AFLD	Procollagen Ⅲ	Propeptide	Released during matrix deposition and remodelling
Boeker[75] Murawaki[62]	HCV	MMP-2	Collagenase	Correlates with fibrosis
Boeker[75]	HCV	TIMP-1	Metalloproteinase	Inhibitor of collagenase
Patel[76]	HCV	Three-marker panel	Combination of hyaluronic acid, TIMP-1, α_2M	Better accuracy by combined markers

AFLD = alcoholic fatty liver disease; NAFLD = non alcoholic fatty liver disease; ECM = extracellular matrix; HSCs = hepatic stellate cells; YKL-40 = human cartilage glycoprotein-39; MMP-2 = metalloproteinase 2; TIMP-1 = tissue inhibitor metalloproteinase 1; α_2M = alfa-2-macroglobulin.

stage of liver fibrosis, with variable results (Tables 6 and Table 7). A second and easier approach in the search of non invasive markers of liver fibrosis has been to take single or combined haematological or biochemical parameters that reflect the stage of liver disease and to assess and compare the accuracy of their diagnostic performance. This approach, that often uses routinely performed blood tests, has led to the identification of sets of markers able to define the stage of liver fibrosis with an accuracy very similar, if not superior, to that of the more sophisticated and difficult to test direct markers. The diagnostic performance of most direct and indirect markers of liver fibrosis has been investigated in all the common etiological forms of CLDs, including hepatitis C, hepatitis B and alcoholic and non alcoholic fatty liver and steatohepatitis, although some of them have been more extensively tested in patients with chronic hepatitis C.

WHAT THE IDEAL NON INVASIVE MARKER OF LIVER FIBROSIS SHOULD IDENTIFY?

Most non invasive markers of liver fibrosis described in the literature were developed with the aim of discriminating between "insignificant" (F0-F1 by METAVIR) and clinically "significant" fibrosis (≥ F2 by METAVIR) or of identifying or excluding established cirrhosis in patients with well compensated CLD. Both these aims are clinically most relevant. Presence of significant fibrosis in the liver is indeed considered as the hallmark of a progressive liver disease and a clear indication for immediate initiation of antiviral therapy in patients with chronic HCV or HBV infection, in agreement with International and National Guidelines and recommendations for the management of these conditions[1,77-78]. On the other hand, patients with F0-F1 usually do not progress or progress much slowly[79-80]. Presence of cirrhosis, even when fully compensated and still clinically occult, indicates the need for specific monitoring of complications related to portal hypertension and to the increased risk of developing hepatocellular carcinoma. Furthermore, patients with HCV or HBV related cirrhosis are less likely to respond to interferon-based antiviral therapy and at higher risk of hepatic decompensation in the case of significant ALT flares when relapsing after therapy withdrawal.

THE DIRECT MARKERS OF LIVER FIBROGENESIS

A list of direct markers of liver fibrogenesis are described in Table 4 while Table 6 and 7 report their diagnostic performance in detecting significant fibrosis (≥ F2 by METAVIR) and cirrhosis, respectively, in the different etiologic forms of CLDs in which they have been evaluated. Hyaluronic acid has been extensively studied in hepatitis C and AFLD and, in more recent years, it has also been tested in smaller cohorts of patients with NAFLD and hepatitis B. Overall, a rather good accuracy of hyaluronic acid in the different studies in discriminating significant from insignificant fibrosis has been reported, with an area under the curve (AUC) ranging from a minimum of 0.78 in NAFLD to an excellent 0.98 in hepatitis B (Table 6). However, the number of patients tested was quite low in both these patients categories, with 75 and 112 cases in two studies on NAFLD (AUC of 0.87 and 0.79, respectively)[65,72] and only 65 patients in the single study conducted in chronic hepatitis B[68]. Further studies are clearly needed, especially in hepatitis B since the accuracy reported by Montazeri *et al* was excellent (0.98 AUC) but should now be confirmed in larger studies. In chronic hepatitis C, the ability of hyaluronic

acid to differentiate minimal-mild from moderate-severe fibrosis has been tested in much larger series of patients. In various cohort studies the AUC values have ranged from 0.82 to 0.92 (Table 6). In a study conducted in 326 patients the AUC was 0.86 and the specificity was 95% for significant fibrosis while the AUC was 0.92 and the specificity was 89.4% for cirrhosis when a cut off level of 110 μg/L was used[74]. However, another cohort study with more than 400 cases has reported an AUC of only 0.73 for significant fibrosis[63]. In the same study, cirrhosis could be excluded with excellent negative predictive value and sensitivity (100%) using a cut off level of 50 μg/L. Similar results were reported in another study of 486 patients in which hyaluronic acid levels < 60 μg/L excluded cirrhosis with 99% negative predictive value[61]. In a smaller study hyaluronic acid performed less well in excluding cirrhosis, with an AUC of 0.85 and 80% negative predictive value[71]. In AFLD the performance of hyaluronic acid for significant fibrosis varied significantly[64,66] while the marker showed very good performance for cirrhosis, with an AUC of 0.93[66]. On the basis of these findings, the greatest clinical utility of hyaluronic acid might be in its ability in excluding cirrhosis. The results of a study conducted in 79 patients with NAFLD were also encouraging, as hyaluronic acid had a 0.92 AUC value for cirrhosis[65]. Further studies with larger series of cases are needed, especially in NAFLD and chronic hepatitis B.

Among the glycoproteins, laminin has been assessed as a non invasive marker mainly for significant liver fibrosis. It showed an overall accuracy of 81% in a detailed study on 243 patients with CLDs[81]. It performed better in AFLD (84% accuracy) than in viral hepatitis (77% accuracy). Another study of 37 patients with chronic hepatitis C showed slightly better performance (AUC = 0.82)[69]. In a recent study conducted on 30 patients with NAFLD laminin showed good performance, particularly when combined with type IV collagen, with 87% accuracy, 100% specificity and positive predictive value[67].

YKL-40 is a recently described glycoprotein that belongs to the chitinase family. It is strongly expressed in human cartilage and human liver. YKL-40 is a relatively new marker of hepatic fibrosis and it has been only preliminarly evaluated in CLDs. It was initially investigated in patients with alcoholic liver disease. In a cohort of 146 heavy drinkers, YKL-40 showed good specificity (88.5%) but poor sensitivity (50.8%)[70]. Better results have been reported in 109 patients with chronic hepatitis C, with 0.81 AUC, 78% sensitivity and 81% specificity[71]. In the same study the accuracy in predicting cirrhosis was however lower, with an AUC of 0.795. Further studies are needed to elucidate the value of this new non invasive marker of liver fibrosis in chronic viral hepatitis and in fatty liver diseases.

Among the collagens, type Ⅳ collagen has been extensively investigated as non invasive marker of liver fibrosis. Type Ⅳ collagen is composed of a major triple-helix, an amino-terminal triple-helix (7s domain) and a carboxy-terminal globular domain. The first two forms of type Ⅳ collagen have been used in clinical studies. Type Ⅳ collagen has been studied in hepatitis C and NAFLD and a good diagnostic performance for significant fibrosis has been reported, particularly in hepatitis C (AUC = 0.83)[69,73]. Murawaki *et al* have focused on the 7s domain and central triple helix domain and found a slightly better accuracy of the former in detecting cirrhosis, with 75% positive predictive value and 92% negative predictive value[73]. The role of 7s domain has also been investigated in 112 patients with NAFLD and its performance has been compared with hyaluronic acid[72]. The results showed a better diagnostic accuracy for type Ⅳ collagen-7s domain (0.828 *vs* 0.797 AUC, respectively). Several studies have also compared the diagnostic performance of type Ⅳ collagen with that of hyaluronic acid in hepatitis C and reported the superiority of the latter marker[62,71]. These findings would indicate no definitive advantage in using type Ⅳ collagen instead of hyaluronic acid in hepatitis C. Data on type Ⅳ collagen in NAFLD are extremely limited and need further evaluation.

Several studies evaluated a possible role of procollagen Ⅲ in hepatitis C and in AFLD. In comparative studies conducted in hepatitis C, procollagen Ⅲ performed less well than type Ⅳ collagen and hyaluronic acid[71,74]. On the other hand, a good accuracy has been described in AFLD (AUC = 0.867), but again it was slightly worse than hyaluronic acid (0.913)[64]. The superiority of hyaluronic acid and type Ⅳ collagen does not allow to recommend the use of procollagen Ⅲ as non invasive marker of liver fibrosis.

Collagenases and their inhibitors have also been proposed as surrogate markers of liver fibrosis. Those reported to have some clinical impact include metalloproteinase 2 (MMP-2) and tissue inhibitor metalloproteinase 1 (TIMP-1)[82]. In a recent study Boecker and colleagues investigated the role of MMP-2 and TIMP-1 as non invasive markers of liver fibrosis in 78 patients with chronic hepatitis C[75]. Both these proteins were measured with two different methods and their diagnostic performance was different. However, with both methods, the performance in detecting cirrhosis was very high, especially for MMP-2 (0.97 AUC). Unfortunately, it has been difficult to obtain good standardisation of the method for routine clinical use.

Measurements of serum cytokines (TGF-β, TNF-β) involved in fibrogenesis have been assessed in a limited number of studies in which cytokines were found to have somehow less value in predicting liver fibrosis compared to the ECM tests[20,83]. Several Authors have tried to combine different direct markers of liver fibrosis. In a cohort study of more than one thousand patients with CLD an algorithm combining hyaluronic acid, procollagen Ⅲ and TIMP-1 has been described[84]. The AUC was discrete for hepatitis C (0.77), good in NAFLD (0.87) and excellent in AFLD (0.94). Another combination panel of matrix markers (hyaluronic acid, TIMP-1 and α_2-macroglobulin) has been tested in a cohort of HCV patients, obtaining an AUC of 0.83 with an accuracy of 75%[76]. The diagnostic performance of this combination panel appears quite similar to those reported for some single ECM components, particularly hyaluronic acid, laminin and YKL-40. Santos *et al* have recently investigated some ECM components in NAFLD showing that a combination of laminin and type Ⅳ collagen (282 ng/mL and 145 ng/mL

Table 5 Indirect non invasive markers of liver fibrosis

Authors	Liver disease	Biomarker	Description	Rationale
Giannini[85]	HCV, NAFLD	AAR	AST to ALT ratio	AST and ALT levels increase with progressive fibro-
Wai[86] Macias[87]	HCV, HIV/HCV	APRI	AST to platelet ratio	Statistical association with liver fibrosis
Forns[88] Macias[87]	HCV, HIV/HCV	Forns' index	Combination of age, platelet, γGT, cholesterol	Statistical association with liver fibrosis
Islam[89]	HCV	GUCI	Combination of AST, INR, platelet	Statistical association with liver fibrosis
Imbert-Bismut[90] Myers[91] Myers[92] Naveau[66]	HCV, HIV/HCV, HBV, AFLD	Fibrotest	Combination of α_2M, ApoA1, bilirubin, γGT, haptoglobin	Statistical association with liver fibrosis
Sud[93]	HCV	FPI	Combination of HOMA-IR, age, cholesterol, AST, alcohol intake	Statistical association with liver fibrosis
Callewaert[94]	CLDs (mostly HCV)	Glycocirrho test	Profiles of serum protein N-glycans	Glycoproteins are produced mainly by hepatocytes

NAFLD = non alcoholic fatty liver disease; AAR = aspartate to alanine aminotrasferase ratio; α_2M = alfa-2-macroglobulin; ApoA1 = apolipoprotein A1; APRI = AST to platelet ratio index; GUCI = Goteborg University Cirrhosis Index; INR = international normalised ratio; FPI = fibrosis probability index; HOMA-IR = homeostasis model assessment of insulin resistance; CLDs = chronic liver diseases. γGT = gamma glutamil transpeptidase.

cuts off, respectively) could individuate patients with significant fibrosis with 100% positive predictive value and specificity[67]. Unfortunately, only 30 patients were included in this study. In conclusion, combination panel of ECM components may improve the diagnostic accuracy of the single markers, particularly in AFLD and NAFLD, while no clear advantage has been so far demonstrated in patients with chronic hepatitis C. Further studies are needed to validate the new combinations of markers recently proposed for AFLD and NAFLD.

THE INDIRECT MARKERS OF LIVER FIBROSIS

One of the main limitation to the clinical use of direct markers of liver fibrosis is that they are not routinely available in all hospital settings. While direct markers of liver fibrosis reflect the process of fibrogenesis, indirect markers satisfy the request for a simple and easy to perform marker. The indirect markers of liver fibrosis are described in Table 5. Their diagnostic performance in detecting significant fibrosis and cirrhosis is reported in Table 6 and Table 7, respectively. The first indirect marker of liver fibrosis were transaminases, later associated in the aspartate to alanine aminotrasferase ratio (AAR) to detect cirrhosis[85]. The strength of such marker is the simplicity and the immediate availability for every Hepatologist and Clinician. On the other side, the numerous studies conducted showed that its accuracy is highly variable[83]. Moreover, it cannot be used to differentiate between no-mild and moderate-severe fibrosis. A further evolution of this index was later introduced by Wai *et al* who combined aspartate aminotrasferase (AST) with platelet count[86]. This AST to platelet ratio index (APRI) was then assessed in several studies conducted with a cohort of patients with hepatitis C and showed a rather good diagnostic performance and reproducibility, particularly for cirrhosis (AUC range from 0.77 to 0.94)[86,95-97]. The real strength of such index is that it is based on blood tests that are routinely performed in patients with liver disease with no need for additional blood collection

Table 6 Diagnostic performance of non invasive markers of liver fibrosis in discriminating between no-mild fibrosis (F0-F1 by METAVIR) and moderate-advanced fibrosis (F ⩾ 2 by METAVIR)

Marker	Disease	Sensitivity	Specificity	AUC	References
Direct markers of liver fibrosis					
Hyaluronic acid	HCV	75-79	80-100	0.82-0.92	[61-62] [69,74]
	HBV	91	98.1	0.98	[68]
	AFLD	87	93	0.79-0.91	[64,66]
	NAFLD	66-85	68-91	0.78-0.87	[65,67] [72]
Laminin	HCV	80	83	0.82	[69,81]
	NAFLD	82	89	n.a.	[67]
YKL-40	AFLD	88.5	50.8	n.a.	[70]
	HCV	78	81	0.81	[71]
Type Ⅳ collagen	HCV	73-80	81-85	0.83	[69,73]
	NAFLD	64	89	n.a.	[67]
Type Ⅳ collagen-7s	HCV	74-83	75-88	n.a.	[73]
	NAFLD	70	81	0.83	[72]
Procollagen Ⅲ	HCV	60-78	74-75	0.69	[71,74]
	AFLD	80	87	0.87	[64]
MMP-2	HCV	7-75	70-100	0.59	[62,75]
TIMP-1	HCV	67	68	0.71	[75]
Three marker panel	HCV	77	73	0.83	[76]
Indirect markers of liver fibrosis					
APRI	HCV	41-91	47-95	0.69-0.88	[86,95-97]
	HIV/HCV	51	91	0.8	[87]
Forns' index	HCV	79.8-94	95-98.3	0.78-0.86	[88,98] [99,97]
	HIV/HCV	43	96	0.77	[87]
Fibrotest	HCV	65-87	59-80.6	0.74-0.87	[90,97] [100,101]
	HIV/HCV	90	60	0.85	[91]
	HBV	34	93	0.78	[92]
	AFLD	88	60	0.84	[66]
FPI	HCV	85-96	94-98	0.77	[93]

AUC=area under the curve; AFLD=alcoholic fatty liver disease; NAFLD=non-alcoholic fatty liver disease; n.a.=not available; YKL-40=human cartilage glycoprotein-39; MMP-2=metalloproteinase 2; TIMP-1=tissue inhibitor metalloproteinase 1; APRI=AST to platelet ratio index; FPI= fibrosis probability index.

or costs. Most recently, APRI has been modified by adding alanine aminotrasferase (ALT) and international normalised ratio (INR), with further improvement of the diagnostic accuracy, particularly for cirrhosis[103]. Another rather simple index has been described by Forns *et al*[88]. It derives from combination of age, cholesterol level, gamma glutamil transpeptidase (γGT) and platelet count and was developed to differentiate no/minimal (F0-F1) from significant (≥ F2) fibrosis in hepatitis C, while it gives no informations on cirrhosis. It has been suggested that Forns' index might be less accurate in patients with HCV genotype 3 that is associated with very low cholesterol levels[99]. A recent study by Macias and colleagues have tested the role of APRI and Forns' index as non invasive markers of liver fibrosis in 357 patients coinfected with human immunodeficiency virus (HIV) and HCV[87]. Overall, the markers showed a lower diagnostic accuracy than in HCV mono-infected patients. The most important limits of both APRI and Forns' index are in the fact that they leave almost half of the patients unclassified.

Another index that combines together standard biochemical serum markers such as AST, platelet count and INR has been reported[89]. It showed good accuracy for cirrhosis in hepatitis C, but without significant improvement with respect to the individual tests used alone. The most widely investigated combination set of non invasive markers of liver fibrosis is Fibrotest. This was initially proposed in 1999 by Imbert-Bismut and colleagues as fibroscore and uses a combination of five blood tests including γGT, bilirubin, haptoglobin, apolipoprotein A1, α2-macroglobulin, adjusted for gender and age[90]. Fibrotest and Fibrosure are the commercially available equivalents of fibroscore in Europe and in the US, respectively. Fibrotest-Fibrosure has the great advantage of classifying all stages of liver fibrosis and it does not leave any patient unclassified. On the other hand, it uses two rather uncommon parameters, apolipoprotein A1 and α2-macroglobulin and requires precise standardisation of the laboratory procedures[104]. To date, Fibrotest-Fibrosure is by far the most investigated and validated non invasive marker of liver fibrosis with around 20 studies reported in the literature. It has been extensively tested in chronic hepatitis C where it shows an AUC of around 0.85 for significant fibrosis. Fibrotest was also tested in chronic hepatitis B and it performed slightly worse, with an AUC of 0.78 in the only study conducted[92]. In the only study performed in HIV/HCV coinfected patients Fibrotest performed well, particularly for cirrhosis (AUC = 0.87) that could be excluded with 100% negative predictive value[91]. The same performance on AFLD was obtained in another study from Poynard's group and the accuracy for cirrhosis was particularly high, with an AUC of 0.95[66]. However, no validation study of Fibrotest-Fibrosure has been conducted in HIV/HCV coinfection, chronic HBV infection and AFLD. A recent comparative study of indirect markers of liver fibrosis conducted in our Unit on 190 patients with chronic hepatitis C tested the performance of APRI, Forns and fibrotest[97]. Fibrotest showed the best accuracy, with an AUC of 0.81 for significant fibrosis and of 0.71 for cirrhosis. The same study was also one of the few reporting data on non invasive markers of liver fibrosis in patients with persistently normal transaminases (PNALT). The three non invasive markers of liver fibrosis were tested in 65 HCV patients with PNALT and APRI showed the best accuracy, with an AUC of 0.77 (Table 8). Available data would suggest that Fibrotest-Fibrosure performs well in detecting the two extremes of the staging range of liver fibrosis (F0-1 and F4), while it might performs somehow less well in the intermediate stage (F2)[20,101]. In some validation studies Fibrotest-Fibrosure was not so accurate as described in the early studies[100]. Fibrotest-Fibrosure has also been evaluated in combination with other markers. Callewaert and colleagues have recently proposed a non invasive marker based on profiles of serum glycoproteins (Glycocirrhotest)[94]. They found that the combination of Fibrotest-Fibrosure and Glycocirrhotest allows identification of cirrhosis with 100% specificity and 75% sensitivity. Another study has recently considered the influence of metabolic factors in the development of fibrosis in hepatitis C and proposed an index that includes assessment of insulin resistance and alcohol consumption. The performance of this approach was however inferior to that of simpler markers (0.77 of AUC) and has not been yet externally validated[93]. Recently a new technology (Fibroscan) that measures liver stiffness has been proposed[105]. The rationale is based on the

Table 7 Diagnostic performance of non invasive markers of liver fibrosis in detecting cirrhosis

Marker	Disease	Sensitivity	Specificity	AUC	References
Direct markers of liver fibrosis					
Hyaluronic acid	HCV	80-100	79-89.4	0.85-0.92	[61,63] [71,74]
	AFLD	99	80	0.93	[66]
	NAFLD	n.a.	n.a.	0.92	[65]
YKL-40	HCV	80	71	0.79	[71]
Type Ⅳ collagen	HCV	60	61	n.a.	[71,73]
Procollagen Ⅲ	HCV	60-77	66-74	0.73	[71,74]
MMP-2	HCV	74-83	96-100	0.97	[75]
TIMP-1	HCV	100	56-75	0.9	[75]
Indirect markers of liver fibrosis					
AAR	HCV	47-81.3	55.3-97		[85,102] [96]
	HIV/HCV	38	77	0.6	[87]
APRI	HCV	38.4-57	86.7-93	0.61-0.94	[86,95-97]
	HIV/HCV	53	89	0.79	[87]
GUCI	HCV	80	78	0.85	[89]
Fibrotest	HCV	13-50	91-98	0.71-0.87	[90,97] [100,101]
	HIV/HCV	100	65	0.87	[91]
	HBV	18	99	0.78	[92]
	AFLD	99	83	0.95	[66]
Glycocirrho test	Most HCV	79	86	0.87	[94]

AUC = area under the curve; AFLD = alcoholic fatty liver disease; NAFLD = non-alcoholic fatty liver disease; YKL-40 = human cartilage glycoprotein-39; MMP-2 = metalloproteinase 2; TIMP-1 = tissue inhibitor metalloproteinase 1; AAR = aspartate to alanine aminotrasferase ratio; APRI = AST to platelet ratio index; GUCI = Goteborg University Cirrhosis Index.

Table 8 Diagnostic performance of APRI, Forns' index, Fibrotest and of sequential algorithms combining the three markers in patients with chronic hepatitis C

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	AUC	Classified patients(%)
Detection of significant fibrosis in chronic hepatitis C with elevated ALT							
APRI	29.7	93.8	95.7	52.7	60.2	0.69	54.1
Fibrotest	65	80.6	80	66.7	72.6	0.81	100
Forns	24.3	98.3	94.7	50.9	57.1	0.79	55.5
Sequential algorithm	100	83.8	92.7	100	94.2	n.a.	100
Detection of significant fibrosis in chronic hepatitis C with PNALT							
APRI	26.9	100	100	56.8	62.7	0.77	74
Fibrotest	58.3	91.3	77.7	80.7	80	0.71	100
Forns	11.5	100	100	52.1	54.9	0.58	56
Sequential algorithm	100	87.5	94.3	100	96.3	n.a.	100
Detection of cirrhosis in chronic hepatitis C							
APRI	38.4	86.7	38.5	86.7	78.1	0.61	54.1
Fibrotest	50	92.9	57.9	90.5	85.9	0.71	100
Sequential algorithm	94.6	95.1	78.3	99.1	95.5	n.a.	100

PPV = positive predictive value; NPV = negative predictive value; AUC = area under the curve; APRI = aspartate aminotrasferase to platelets ratio; Forns = Forns' index; n.a. = not available; PNALT = persistently normal ALT.

correlation between liver fibrosis and stiffness. This new method of measuring liver fibrosis has been evaluated in a prospective study conducted in more than seven hundred patients with CLDs of different aetiologies obtaining an AUC ranging between 0.8 for detecting significant fibrosis to 0.96 for diagnosing cirrhosis[106-107]. Fibroscan has also been combined with Fibrotest-Fibrosure in a series of patients with hepatitis C obtaining an AUC of 0.88[108]. The limitation of this new technology is that it requires a costly device to measure liver stiffness, on the other hand it showed great accuracy for cirrhosis. A detailed description of the several studies recently published on the use of Fibroscan in assessing fibrosis in patients with CLDs is not within the purposes of this review aimed to discuss non invasive diagnosis of liver fibrosis by biochemical markers and blood tests.

CURRENT USE OF NON INVASIVE MARKERS OF LIVER FIBROSIS IN CLINICAL PRACTICE AND FUTURE PROSPECTIVES

Although several non invasive markers of liver fibrosis have been developed in the last decade, their implementation in clinical practice has been slow and is still limited. According to many experts in the field, both liver pathologists and clinical hepatologists and also to the most recent international guidelines and recommendations, inter-laboratory variability, lack of reproducibility and, most importantly, an expected rate of misdiagnosis of at least 20% do not yet allow to recommend the use of most of these methods in substitution of liver biopsy[20,78,109]. One of the major limitations may be in the lack of reliable identification and classification of the intermediate stages of fibrosis[83]. On the other hand, from a clinical and not merely statistical point of view what the hepatologist needs is a diagnostic tool that, being on one side non invasive and therefore much more acceptable by the patient compared to liver biopsy, should however ensure not to misdiagnose fibrosis and particularly not to underestimate the stage of fibrosis and presence of cirrhosis. With currently available non invasive tests, this goal cannot be achieved in all patients. The most rational way of using them is therefore that of a compromise in which non invasive markers are first used to classify those patients in which they perform with high accuracy, limiting liver biopsy to the subset in which precise non invasive staging is not possible. Obviously, the indication to take or not to take liver biopsy in the cases in which non invasive markers perform less well will also depend on the need of obtaining a more or less accurate definition of the exact stage of fibrosis and will therefore vary according to the patient's characteristics. As an example, in the elderly population with chronic HBV or HCV infection or fatty liver distinction between minimal and advanced fibrosis may be sufficient for clinical decision independently of obtaining a more precise semiquantitative staging of fibrosis. On the other hand, more precise staging may be required in other patients categories such as those with chronic HBV or HCV infection who are candidate for antiviral treatment, since the decision to start therapy and also the type of drugs to be used may be influenced by fibrosis stage.

The use in clinical practice of non invasive markers of liver fibrosis will most likely increase in the near future as they become more validated and the indication for their selective use in specific patients categories is better clarified and standardised.

SEQUENTIAL ALGORITHMS OF NON-INVASIVE MARKERS OF LIVER FIBROSIS REDUCE THE NEED FOR LIVER BIOPSY IN HEPATITIS C

Recently we have proposed new combination algorithms of non invasive markers for assessing liver fibrosis in

chronic hepatitis C[97]. This represents the first application of a panel of markers used sequentially. Three different algorithms were developed by combining APRI, Forns' index and Fibrotest (Table 8). The rationale was that each individual test has advantages and limitations. APRI and Forns' index leave many patients unclassified while Fibrotest is more expensive and uses two uncommon parameters. Furthermore, the diagnostic accuracy of these methods does not exceed 80%-85% when they are used individually. In the first algorithm, significant fibrosis (≥ F2 by METAVIR) was identified in patients with elevated transaminases with high diagnostic performance (> 94% accuracy) using APRI as screening test, followed by Fibrotest in APRI non-classified cases and restricting liver biopsy to patients classified F0-F1 by non-invasive tests. In the second algorithm excellent accuracy (95%) in identifying cirrhosis was achieved using a similar algorithm with different cut-off levels, limiting by 60%-70% the need of liver biopsy. This marked reduction in the need of taking a liver biopsy and the fact that our algorithm restrict this invasive procedure to patients with low chance of having cirrhosis (being classified as F0-F1 by non invasive markers) is particularly important since the risk of liver biopsy complications is increased in the presence of cirrhosis. We have also developed an algorithm for identifying patients with significant fibrosis among HCV carriers with PNALT. This category has not been considered in most previous studies of non-invasive markers of fibrosis. However, there is abundant evidence in the literature that around 15%-30% of them may have significant fibrosis and a definitive indication to antiviral therapy[110], particularly when considering the favourable results recently reported in such patients with PEG-interferon alfa-2a plus ribavirin combination therapy[111]. The algorithm we have developed in this specific subset of patients reduces by 50% the number of liver biopsies, and shows 93%-95% accuracy in detecting or excluding significant liver fibrosis. However, this subgroup of patients remains difficult-to-diagnose with non invasive markers and liver biopsy is still necessary in around 50% of the cases.

CONCLUSION

Many biomarkers of liver fibrosis have been recently proposed with the aim of substituting liver biopsy. The evidences of the literature on these markers are consistent in showing that: (1) the direct markers of liver fibrosis may have a value in excluding cirrhosis, particularly in hepatitis C (hyaluronic acid, MMP-2), and in predicting fibrosis in NAFLD and hepatitis B (hyaluronic acid, type collagen Ⅳ) but further studies are needed especially in the last two patients subgroups; (2) the indirect markers of liver fibrosis may have a value in excluding HCV-related cirrhosis (APRI, Fibrotest), HCV-related cirrhosis with HIV coinfection and cirrhosis related to AFLD (Fibrotest) but further studies are needed in large cohorts of patients; (3) combination panels of non invasive biomarkers may improve the accuracy of the single tests (algorithm combining hyaluronic acid, procollagen Ⅲ and TIMP-1 in patients with AFLD, combination of laminin and type Ⅳ collagen in NAFLD) but further external validation is needed; (4) a series of algorithms based on sequential combination of non invasive biomarkers have shown high diagnostic accuracy in identifying significant fibrosis and cirrhosis in patients with chronic hepatitis C with a reduction by more than 50% in the need of taking liver biopsies; (5) based on these findings, it is conceivable to anticipate that non invasive markers of fibrosis will become in the near future an important tool in clinical practice; however, implementation of these tests in the diagnostic management of CLDs is expected to reduce but not completely abolish the need for liver biopsy; (6) for future research, priority should be given to large scale validation studies of the most promising non invasive markers and of their combinations in the different forms of CLDs accompanied by progressive fibrosis. There is also an urgent need for better assessment and validation of direct fibrogenesis markers that could be implemented in the prognostic evaluation and in dynamic testing of the efficacy of antifibrotic interventions and treatments.

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REVIEW

Platelet-activating factor in liver injury: A relational scope

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Abstract

The hepatocyte, the main cellular component of the liver, exhibits variable susceptibility to different types of injury induced by endogenous or exogenous factors. Hepatocellular dysfunction or death and regeneration are dependent upon the complicated interactions between numerous biologically active molecules. Platelet-activating factor (PAF) seems to play a pivotal role as the key mediator of liver injury in the clinical and experimental setting, as implied by the beneficial effects of its receptor antagonists. A comprehensive up-to-date overview of the specific functional and regulatory properties of PAF in conditions associated with liver injury is attempted in this review.

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Key words: PAF; PAF-R antagonists; Liver; Injury; Regeneration

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INTRODUCTION

Platelet-activating factor (PAF) (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent pro-inflammatory lipid mediator[1,2] presenting a very broad spectrum of biological activities, including allergic reactions, inflammatory conditions, cardiovascular and neuronal function, reproduction, ischemia-reperfusion (IR) organ injury, sepsis, shock and tumorigenesis, which are being continuously populated[3]. PAF is typically produced by several types of leukocytes (i.e. basophils, eosinophils, neutrophils, macrophages and monocytes), platelets, endothelial cells and many other types of stimulated cells and has been detected in virtually all biological fluids. PAF exerts its activity through binding to a specific receptor, PAF-R, a seven transmembrane, heterotrimeric G protein-linked membrane receptor[4]. PAF-R transduces pleiotropic functions such as cell motility, smooth muscle contraction, synthesis and release of mediators and cytokines[5]. It has also been speculated, supported by the observation that most of the synthesized PAF remains within the cell, that PAF possibly plays an intracellular role in addition to its function as an extracellular communication molecule[6]. Moreover, PAF may play a role in many pathophysiological processes as a cofactor, i.e. as a distinct component of a complex mediator network. PAF is enzymatically synthesized through two major routes, the so-called remodeling and *de novo* pathways, with the contribution of an acetyltransferase (PAF-AT) and is degraded by acetylhydrolases (PAF-AH) into its biologically inactive form, lyso-PAF[7]. The biochemical steps of biological PAF synthesis and degradation are schematically presented in Figure 1. PAF-AHs exhibit phospholipase A_2 (PLA_2)-like activity leading to hydrolysis of the *sn2* ester bond, reversing the acetyltransferase synthetic step and generating lyso-PAF and acetate. One plasma-type and two intracellular PAF-AH have been described and cloned[8]. Plasma PAF-AH is a monomeric enzyme synthesized and secreted by macrophages (and to a lesser extent in the liver) and associated with lipoproteins in plasma. Intracellular PAF-AHs are divided into types I and II, with type II PAF-AH showing a broader specificity and significant homology to the plasma-type enzyme. PAF-AHs obviously control, through elimination or inhibition, PAF activity in cases of excessive production and release of this potent mediator[9], although their precise role in normal and disease state is still poorly understood.

PAF has been suspected to play an important role in liver pathophysiology, particularly associated with inflammatory conditions. Its contribution as a mediator to the pathogenesis of liver injury in regenerating livers, through activation of multiple intermediate molecules or cofactors, has been elucidated in several experimental studies. In the liver, PAF is mainly produced and released by Kupffer cells facilitating communication and interaction between hepatic sinusoidal and parenchymal cells. The regulatory role of PAF in leukocyte recruitment, microvascular dysfunction and cytokine production associated with liver injury remains a main target of current research.

This review aims to present in a collective way the information available concerning the involvement of

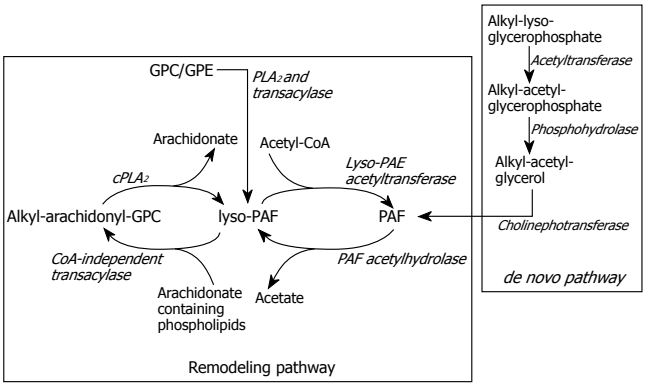


Figure 1 Remodeling and *de novo* pathways of platelet-activating factor (PAF) synthesis. GPC = Glycerophosphocholine; GPE = Glycerolophosphoethanola mine; Lyso-PAF = Alkyl-lyso-GPC; PAF = Alkyl-acetyl-GPC; $cPLA_2$ = Cytosolic phospholipase A_2.

PAF in various types of liver injury in order to reveal its crucial role in liver pathophysiology. The already reported effects of specific PAF-R antagonists on liver injury and regeneration are also mentioned.

PAF AND LIVER INJURY

PAF and hepatic ischemia-reperfusion (IR) injury

Hepatic ischemia-reperfusion (IR) injury is a common issue encountered in various clinical conditions, including systemic shock followed by hepatic failure, liver transplantation and liver resections or extensive hepatectomy due to trauma or cancer. Although hepatocellular injury does occur during the period of ischemia or hypoperfusion of the liver, there is increasing evidence that most of the hepatic cellular damage occurs during reperfusion of the ischemic liver, supporting the assumption that hepatic injury due to ischemia is accentuated after the re-establishment of oxygen flow[10]. Among the mechanisms accounting for this type of liver injury, which include oxygen-derived free radical formation, cellular energy depletion leading to cell membrane dysfunction[11], disruption of calcium homeostasis[12], activation of phospholipases[13], production and release of soluble mediators such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF)-α, neutrophil stimulation, chemoattraction and adhesion to activated endothelial sites resulting in microvascular injury[14-16], PAF is thought to play a major role as a mediator of the inflammatory events following hepatic IR[17]. Several experimental animal models have been used to investigate the extent of cellular disruption and the protective mechanisms involved in liver IR injury in association with PAF activity and the potentially beneficial effects of potent PAF-R antagonists. The variability of these models remains a major issue limiting the reliable assessment and comparison of their results. Studies conducted as yet can be schematically divided into two groups: *in situ* and isolated hepatic IR studies. Table 1 summarizes the features of experimental studies using PAF-R antagonists.

Liver function following hepatic IR has been initially assessed in studies using an isolated liver perfusion apparatus. In an early study[18] comparing cold liver preservation in the Eurocollins and the University of Wisconsin (UW)-lactobionate solutions, the efficacy of pretreatment with SRI-63441, a potent PAF-R antagonist, was also evaluated. Livers isolated from pretreated rats and stored in the UW solution for 24 h produced significantly more bile than livers preserved in the UW solution alone. Although the differences in other parameters of liver function evaluated in this study did not reach statistical significance, the observed beneficial effect was attributed to PAF antagonism, supporting the assumption that PAF was implicated in cold ischemic liver injury. The role of PAF and its antagonist CV-6209 in cold ischemic liver injury of similar duration has also been investigated[19]. After 24 h of cold preservation, bile flow was significantly higher in all CV-6209-treated groups at two hours of reperfusion and at specific time points with a dose-dependent recovery outcome, which also correlated with significantly lower aspartate aminotransferase (AST) levels. A tendency toward lower liver weight increase and lower perfusion pressures was also noted. Using the same isolated liver perfusion system, an experimental series of warm liver ischemia revealed better recovery of bile flow in groups which received higher doses of CV-6209 over a specific threshold of antagonism. In addition, AST level increase was significantly lower within certain dosage limits and a tendency toward lower perfusion pressures was observed. These results suggested that PAF was possibly implicated in both warm and cold ischemic liver injuries, which might be effectively diminished by a potent receptor antagonist. SRI-63441 has also been used in another series of isolated liver perfusion experimental studies[20] aiming to evaluate the pharmacologic modulation of warm postischemic hepatic function. Pretreatment with this PAF-R antagonist before the induction of *in situ* total hepatic ischemia resulted in significantly increased bile production, decreased perfusate transaminase levels and higher tissue adenosine triphosphate (ATP) content compared to ischemic non-treated controls. The degree of hepatocellular vacuolization and sinusoidal endothelial disruption due to ischemic injury revealed by electron microscopy was also less severe in the pretreatment group. These findings confirmed the dose-dependent protective effect of this antagonist against warm IR injury, thus implicating PAF as a key mediator of this type of liver injury. The direct effect of PAF on ischemic liver injury has been investigated in an *ex vivo* isolated rat liver perfusion system[21], in order to avoid the interference of other factors. Administration of high doses of PAF, expected to result in shock and death *in vivo*, did not exacerbate hepatic ischemic injury in this model after 30 or 60 min of ischemia. This finding suggested that PAF, unlike its *in vivo* action, neither causes hepatocellular injury nor enhances ischemic liver injury directly, supporting the hypothesis that PAF exerts its effects through other factors and substantially acts as a key mediator of ischemic liver injury. However, in another similar study, administration of increasing doses of PAF significantly decreased liver tissue ATP levels and oxygen consumption and significantly increased alanine aminotransferase (ALT) and purine nucleoside phosphorylase (PNP) activity in the effluent perfusate compared to control values[22]. The noted disruption of

Table 1 Experimental studies of IR liver injury evaluating the role of PAF through its antagonism

Ischemia				Reperfusion		
Type of ischemia	Splanchnic congestion	Duration	PAF-R antagonist	Type of reperfusion	Duration	Reference
is, T, W	No	120 min	TCV-309	is	1, 3, 6 h - s 7 d	[34]
is, T, W	No	120 min	TCV-309	is	30 min, 1, 3, 6 h - s 30 d	[33]
is, T, W	No	120 min	TCV-309	is	60 min - s 30 d	[32]
is, T, W	Yes	20 min	E5880	is	30 min	[31]
is, T, W	Yes	45 min	BB-882 (lexipafant)	is	45 min	[35]
is, T, W	NA	120 min	WEB-2170	is	6, 24 h	[37]
is, T, W	NA	60 min	BN-52021	is	60 min	[38]
is, P, W	No	60 min	WEB-2170	is	1, 3, 6, 24 h	[24]
is, P, W	No	30, 45 min	WEB-2170	is	1 h	[28]
is, P, W	No	60 min	BN-52021	is	30 min	[27]
is, P, W	No	60 min	BN-52021	is	30 min	[29]
is, P, W	No	75 min	BN-52021	is	24 h - s 7 d	[26]
is, P, W	No	30, 60, 90 min	CV-6209	is	15, 30, 60, 120 min	[23]
Is, T, W+C	NA	90 min (W), 24 h (C)	CV-6209	Is	60 min (W), 120 min (C)	[19]
is, T, W	NA	90 min	SRI-63441	Is	90 min	[20]
Is, T, C	NA	24 h	SRI-63441	Is	90 min	[18]

is: *In situ*; Is: Isolated; T: Total; P: Partial; W: Warm; C: Cold; NA: Not applicable; s: Survival; d: Days.

parenchymal cells in association with the unaltered PNP/ALT ratio, used as a relative indicator of non-parenchymal cell injury, suggested that PAF might lead to functional impairment of parenchymal hepatic cells, i.e. hepatocytes, even in the absence of microcirculatory disturbance secondary to the interaction between circulating leukocytes and endothelial cells.

Experimental studies of *in situ* hepatic IR have consistently used models of total, with or without splanchnic congestion, or partial liver ischemia. Partial liver ischemia by clamping or ligation of the hilar area of the left lateral and median rat liver lobes, thereby avoiding intestinal congestion, has been extensively applied in several warm hepatic IR studies. Demonstration of the protective effects of CV-6209, in terms of improved bile flow and earlier recovery of rat liver ATP levels and energy charge after 60 min of ischemia, as well as determination of the optimal timing and dosing pattern were attained in a leading study conducted by Wang *et al*[23]. The contribution of PAF to the inflammatory consequences of hepatic IR injury, especially during the late-phase hepatic reperfusion, has been evaluated[24] by measurement of hepatic PAF levels[25] and intraperitoneal administration of the PAF-R antagonist WEB-2170. Treatment with WEB-2170 resulted in a significant decrease of plasma ALT levels and polymorphonuclear (PMN) leukocytes infiltration in liver tissue in association with impaired hepatocellular necrosis compared to the control group. Additional pharmacological interventions provided evidence that interaction between Kupffer cells and oxygen-derived free radicals led to local hepatic generation of PAF after IR. A survival study[26] using a partial hepatic IR injury model demonstrated that pretreatment with the PAF-R antagonist BN-52021 resulted in a statistically significant reduction of warm liver injury in terms of improved seven-day cumulative survival rates and histological evidence of minor hepatocellular necrosis on the first postoperative day and revealed a dose-dependent protective effect. Pre-ischemic administration of BN-52021 in a similar hepatic IR model[27] resulted in a significant reduction of hepatocellular loss of cytoplasmatic ALT and intramitochondrial glutamate dehydrogenase (GLDH) into the plasma. In this study, significantly reduced endothelial enzyme leaking (PNP), increased bile flow and liver tissue ATP content and moderately decreased malondialdehyde (MDA) levels in the pretreated reperfused livers were observed, suggesting the implication of PAF in both structural and metabolic changes induced by hepatic IR injury. Microcirculatory disruption due to hepatic IR has also been investigated[28]. Pretreatment with WEB-2170 did not result in significant recovery of postischemic liver blood flow and liver oxyhemoglobin saturation, as determined by laser Doppler flowmetry and photometry, respectively. It was therefore assumed that the involvement of PAF might be secondary in the pathogenesis of reperfusion injury or bypassed by other major contributors, such as leukocytes in this instance. Nevertheless, another contemporary study evaluating the role of PAF in microcirculatory disturbance in a similar experimental model[29] demonstrated that pretreatment with BN-52021 resulted in significant improvement of left lobular erythrocyte flux recovery, reaching nearly preischemic control values after 10 min of reperfusion. In addition, PNP leakage from hepatic vascular endothelium into plasma as a result of reperfusion was significantly attenuated by administration of BN-52021. A statistically significant reduction of postischemic hepatocellular ALT loss, accompanied by enhanced bile production and increased tissue ATP levels as well as by suppressed neutrophil infiltration in liver tissue, as indicated by reduced myeloperoxidase (MPO) activity, were also observed in the pretreatment group, suggesting that PAF might be implicated in IR-induced microcirculatory disturbance. The emerging controversy between these studies seems to have been resolved by a recent experimental evaluation of the effects of PAF and a thromboxane (Tx)-A_2 analogue

on hepatic vascular resistance distribution and liver weight in an isolated perfused animal model[30]. PAF was shown to be a potent vasoconstrictor causing a dose-dependent increase of total hepatic vascular resistance. Furthermore, it was demonstrated that PAF increases predominantly presinusoidal over post-sinusoidal resistance and influences liver weight, causing liver weight loss at low concentrations and liver weight gain at high concentrations. These findings support the presumption that PAF is involved in the induction of hepatic microcirculatory disturbance in conditions such as IR injury.

In many recent studies, total hepatic ischemia has been applied using the Pringle's maneuver, i.e. by ligation or cross-clamping of the portal triad - hepatic artery, portal vein and bile duct. The effects of pretreatment with the PAF-R antagonist E5880 on hepatic and systemic metabolism have been investigated after a relatively short period of *in situ* total warm hepatic ischemia followed by a similarly short period of reperfusion, without avoiding intestinal congestion[31]. In the pretreatment group, hepatic tissue ATP and energy charge levels were significantly increased while AMP levels were significantly decreased after 30 min of reperfusion compared to non-pretreated animals. However, no statistically significant difference was noted in parameters reflecting hepatic mitochondrial function, such as the arterial ketone body ratio (AKBR - ratio of acetoacetate to 3-hydroxybutyrate) and arterial ammonia levels, or those indicating systemic changes, such as arterial blood gas values and pyruvate and lactate levels. These data suggested that PAF exerts local effects during liver IR injury, attenuated by its antagonism, without mediating systemic metabolic changes. The effects of the specific PAF-R antagonist TCV-309, alone or in combination with a prostaglandin I_2 analogue, on total warm hepatic IR injury were evaluated in a large study[32]. After two hours of *in situ* hepatic ischemia followed by one hour of reperfusion, improvement of bile flow rate, suggesting restoration of hepatic ATP levels, a significant decrease of tissue glutathione disulfide/glutathione (GSSG/GSH) ratio and less severe midzonal liver injury and congestion were observed in groups treated with TCV-309, indicating preservation of hepatic function and attenuation of oxidative stress. Despite a significant increase in portal endotoxin levels in pretreated animals, 30 d survival rates were higher compared to non-treated groups. These results suggested that PAF was generated in the liver during warm IR leading to liver injury. Using the same PAF-R antagonist, a similar study focusing on cytokine production and neutrophil activation induced by PAF after hepatic IR was conducted[33]. Total hepatic ischemia was produced while intestinal congestion was avoided using an extracorporeal portosystemic shunt. During the reperfusion period, an increased 30-d survival rate, decreased plasma AST levels, significant suppression of plasma TNF-α and CINC (cytokine-induced neutrophil chemoattractant) levels, less severe hepatic necrosis, markedly decreased neutrophil accumulation and significantly lower O_2^- production in liver tissue were noted in the pretreatment group compared to the control group. Inhibition of TNF-α and CINC production by the specific PAF-R antagonist TCV-309 strongly suggested that PAF functions on hepatic IR injury were exerted through either direct neutrophil activation or cytokine induction. The role of this specific PAF-R antagonist in hepatic IR injury has been further evaluated in a recent survival study of total hepatic ischemia[34]. Pretreatment with TCV-309 significantly suppressed the increase in plasma levels of AST and endothelin (ET)-1, a potent vasoconstrictor derived from vascular endothelium and hepatic sinusoidal endothelial cells which leads to hepatic microvascular injury, at all selected time points after reperfusion. In addition, at 6 h after reperfusion, hepatocellular necrosis and neutrophil accumulation in midzonal and pericentral areas of the hepatic lobule were less prominent in TCV-309-treated group. Similarly, survival rates demonstrated a statistically significant improvement in TCV-309-treated animals compared to controls. The complicated interaction between PAF and ET-1 in hepatic IR injury evaluated in this study supported the hypothesis that PAF-R antagonism might exert a potential protective effect against this type of injury via indirect modulation of plasma ET-1 levels. A most recent study of total hepatic IR[35] showed that pretreatment with the PAF-R antagonist BB-882 (lexipafant) resulted in a statistically significant decrease of liver damage scores[36], the mean hepatic MDA levels (sensitive indicator of lipid peroxidation) and serum AST, ALT and lactate dehydrogenase (LDH) levels. These beneficial effects were attributed to a reduction in the inflammatory reaction and oxygen radical synthesis induced by PAF after hepatic IR injury. In order to confirm the putative etiologic relation between PAF, xanthine oxidase, reactive oxidants and leukocytes in the pathogenesis of liver injury, a modified hemorrhagic shock-resuscitation rat model based on Wigger's isobaric principle has been used[37]. In this unique study, inhibition of PAF-R binding by WEB-2170 significantly attenuated leukocyte accumulation in the pericentral areas of the hepatic lobule, both at the early inflammatory response time point and at 24 h end point, as demonstrated by total hepatic MPO activity and histochemical examination. Significantly less severe hepatic injury, evident by plasma AST and alcohol dehydrogenase (ADH) levels and histological grading of hepatocellular necrosis, was also observed in the WEB-2170-treated group, although there was no decrease in the generation of hepatic oxidant stress, as shown by hepatic reduced GSH and oxidized GSSG levels. These results, in combination with those yielded from the administration of other potentially protective factors, established an apparent etiologic relation between PAF and the aforementioned components of hepatic injury *in vivo*. In another experimental model of hemorrhagic shock, causing whole-body IR, the effects of PAF on calcium homeostasis were investigated[38]. PAF is known to use voltage- and receptor-gated Ca^{2+} influx to exert its actions in a variety of cell types[5]. In this model, hepatocellular Ca^{2+} uptake, initial rate of Ca^{2+} influx and membrane Ca^{2+} flux, determined using specific incubation techniques[39], were significantly increased in the shock group compared to sham-operated animals. Administration of the specific PAF-R antagonist BN-52021 in the shock group significantly reduced Ca^{2+} uptake, without influencing other parameters of calcium homeostasis, and largely prevented hepatocyte lipid peroxidation, determined fluorometrically[40]. These results suggested that PAF may play a pivotal

Table 2 PAF and liver transplantation

Type of study	PAF-R antagonist used	Alterations in biochemical parameters of liver graft function (serum)[1]	Reference
Clinical			
	-	[2]	[43]
	-	[2]	[44]
Experimental			
	BN-52021	↓ LDH	[45]
	WEB-2170	NS	[47]
	WEB-2086	[2]	[48]
	E5880	↑ AKBR, ↓ AST, ↓ ALT, ↓ lactate	[46]
	E5880	↑ AKBR, ↓ AST, ↓ ALT, ↓ lactate	[49]
	E5880	↓ AST, ↓ lactate	[50]

[1] Only statistically significant alterations are included in the table above; [2] Not determined; NS = Not significant; LDH = Lactate dehydrogenase; AKBR = Arterial ketone body ratio; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase.

role in promoting hepatocyte Ca^{2+} overload during hemorrhagic shock, which in turn accentuates hepatic oxidative injury.

PAF and liver transplantation

In the field of clinical liver transplantation, liver injury due to cold ischemia followed by warm reperfusion, referred to as preservation-reperfusion injury (PRI) of the graft, remains one of the most critical issues affecting postoperative graft viability and ultimate survival of the recipient. It has been well established that hypothermic ischemia mainly causes sinusoidal lining cell injury which becomes fully manifest on reperfusion, leading to deterioration of the microcirculation[41]. Although the exact mechanisms have not yet been fully elucidated, PAF has been proposed as a major mediator involved in the pathogenesis of PRI and several studies have attempted to clarify the potentially protective role of PAF-R antagonists, as shown in Table 2.

A clinical study of 17 patients who underwent orthotopic liver transplantation (OLT)[42] revealed that peripheral blood PAF levels were not significantly altered during the immediate reperfusion period or during the acute rejection episodes, confirming that successful OLT normalizes blood PAF levels in cirrhotic decompensated patients and that PAF levels were probably unrelated to the onset of acute graft rejection. On the other hand, in another clinical study, PAF levels were increased after portal venous reperfusion, except for one case where veno-venous bypass was applied, suggesting that PAF was probably involved in postoperative liver graft dysfunction[43].

Experimental studies evaluating the role of PAF in liver transplantation through its antagonism have consistently used porcine or rat models. In a rat OLT model, administration of BN-52021 in the preservation solution and to the recipient prior to liver graft implantation[44] resulted in significant reduction of serum LDH and PNP levels and significant improvement of hepatic bile production after reperfusion in the treated group compared to untreated controls. A tendency toward suppressed oxygen free radical generation, as indicated by attenuation of serum conjugated dienes (CD) levels, and liver tissue-associated MPO activity was also noted in the treatment group, although the differences were not statistically significant. The potentially protective effect of the potent PAF-R antagonist E5880 on PRI of the liver graft has been evaluated in another OLT study[45]. Survival rates after 12 h of reperfusion, AKBR, an accurate marker of liver graft metabolic function, and white blood cell (WBC) count were significantly higher, whereas AST and ALT levels at 4 h and lactate levels were lower in the treatment group compared to controls. Histological examination revealed only occasional presence of inflammatory cells, mainly PMNs, in the sinusoids of the treatment group. These results confirmed the implication of PAF in PRI as well as the protective effect of its antagonism. A similar study demonstrated that treatment with WEB-2170 resulted in suppressed, though not statistically significant, postoperative elevation of hepatocellular enzymes (AST, ALT) and bilirubin levels in hepatic venous blood samples and in normalization of local tissue oxygen tension after 6 h of reperfusion, as determined by the use of a multiwire oxygen electrode on liver surface[46]. These data suggested that PAF antagonism attenuated hepatocellular injury and prevented the detrimental shifts of local tissue oxygen tension which reflects the integrity of hepatic microcirculation. The role of PAF as a mediator involved in the regulation of leukocyte adhesion after OLT has been evaluated through administration of the PAF-R antagonist WEB-2086[47]. In the treatment group, the temporary leukocyte adhesion to sinusoidal endothelial cell lining in the periportal region, determined by the method of intravital fluorescence microscopy, was significantly reduced in the early reperfusion period followed by a similar reduction of permanent leukocyte adhesion in the late one. Comparable changes in midzonal and pericentral areas were also noted. Suppression of leukocyte adhesion by this PAF-R antagonist was further reflected by an increase in peripheral blood WBC count during the early reperfusion period. These results suggested that PAF antagonism attenuated the inflammatory response in hepatic sinusoids, thus contributing to the preservation of microcirculation, whereas no protective effect on hepatocellular integrity was noted. The effects of E5880 on PRI of the liver graft in porcine OLT were further assessed in a survival study[48], in which postoperative survival of more than 12 h was significantly higher in the PAF-R antagonist treatment group. Treated animals demonstrated a slight but significant increase in WBC count at 2 h after reperfusion and at 4 h AST, ALT and lactate levels were significantly lower compared to the control group. In addition, the AKBR was significantly increased at 12 h after reperfusion, indicating better recovery of liver graft function. Histological examination by light and electron microscopy revealed less severe sinusoidal endothelial cell damage in the treatment group. Similar results were obtained in another study in which prolonged warm hepatic ischemia in non-heart-beating donors was followed by cold preservation and reperfusion[49]. Improved survival rate at 12 h after OLT, suppression of serum AST and lactate levels, attenuation of hepatocellular degeneration and significantly better energy charge recovery were noted in animals treated with E5880 compared to controls. Although most parameters

Table 3 PAF and acute liver injury

Method	PAF levels	PAF-R antagonist used	Reference
LPS/*P.acnes*	[1]	•	[51]
LPS/PAF	[1]	BN-52021, WEB-2170	[52]
	[1]	WEB-2086	[53]
	[1]	triazolam	[54]
LPS	[1]	WEB-2086	[56]
	[1]	BN-50739, WEB-2170	[59]
	[1]	WEB-2086	[61]
	[1]	TCV-309	[62]
	[1]	BN-50739, WEB-2170	[63]
D-GalN/LPS	[1]	WEB-2086	[64]
	[1]	WEB-2086	[65]
ANIT	[1]	WEB-2086	[66]
APAP	↑ (serum)	-	[67]
	↑ (serum)	BN-52021	[68]
BDL	↑ (plasma, tissue)	-	[69]
	↑ (tissue)	BN-52021, WEB-2170	[70]
	[1]	-	[71]
70% PH	[1]	-	[72]
	[1]	TCV-309	[73]

[1] Not determined; LPS = Lipopolysaccharide; D-GalN = D-galactosamine; ANIT = Alpha-naphthylisothiocyanate; APAP = Acetaminophen; BDL = Bile duct ligation; PH = Partial hepatectomy.

did not reach statistical significance, this was partly attributed to the small number of treated animals.

PAF and acute liver injury

Acute hepatic dysfunction, or even failure, occurs frequently in the clinical setting and is usually refractory to currently available treatment options. The pathogenesis of hepatocellular damage has been well characterized in several experimental studies of acute liver injury induced by lipopolysaccharide (LPS) compounds, chemical and pharmacological factors, bile duct ligation or hepatectomy (Table 3).

In an early study of endotoxin-induced liver injury by intravenous infusion of heat-killed *Propionibacterium acnes* and LPS[50], treatment of experimental animals with a PAF-R antagonist attenuated liver tissue damage, as indicated by the reduction of focal liver necrosis in histological examination. In addition, liver adherent cells isolated from control and endotoxin-treated mice were capable of producing PAF upon stimulation with A23187 calcium ionophore. These results suggested that the produced PAF by liver adherent cells could be involved in the induction of liver injury during endotoxemia. The effects of endotoxin and PAF in rats with secondary biliary cirrhosis were also evaluated[51] and it was suggested that the hemodynamic changes observed in cirrhosis might be aggravated during acute infections. LPS administration significantly increased heart rate and total peripheral vascular resistance and reduced cardiac index, portal pressure and renal blood flow in cirrhotic animals compared to controls. PAF infusion resulted in similar changes in the experimental group, with the exception of a sharp rise in portal pressure owing to an increase in portal territory vascular resistance. Treatment with the specific PAF-R antagonists WEB-2170 or BN-52021 largely prevented LPS-induced renal vasoconstriction and the former also restored cardiac index and peripheral resistance to near normal levels. The complicated interactions between LPS, PAF and TNF-α have been further elucidated[52]. Administration of either LPS or PAF resulted in elevated serum TNF-α levels and TNF-α mRNA production in the intestine and the liver in response to LPS was only partially inhibited by the PAF-R antagonist WEB-2086. These results suggested that LPS induces TNF-α production via both PAF-dependent and PAF-independent pathways and that these major inflammatory mediators interact through a positive feedback mechanism. LPS-associated activation and PAF-induced biochemical response of hepatic macrophages and endothelial cells have also been investigated[53]. PAF administration resulted in a rapid and transient increase in intracellular calcium content in both cell types whereas a rapid decrease in intracellular pH of hepatic macrophages only, attenuated by the PAF-R antagonist triazolam, was also noted. In addition, LPS caused a significant increase in the number of PAF-binding sites/cell and the equilibrium dissociation constant (Kd) for PAF in endothelial cells. These results revealed that the biochemical responses of these two major hepatic cell types to PAF during endotoxemia are distinct. In a unique model of LPS-induced liver injury, based on evidence that intestinal IR increases portal and systemic plasma endotoxin levels[54], the role of Kupffer cells in mediating leukocyte recruitment, impaired sinusoidal perfusion and liver tissue hypoxia via complicated interaction with PAF and TNF-α was investigated[55]. In this study, treatment with WEB-2086 effectively attenuated LPS-induced leukostasis in all segments of hepatic microvasculature, the number of nonperfused sinusoids and elevation in plasma ALT and TNF-α levels. Furthermore, the increased autofluorescence of pyridine nucleotide [NAD(P)H], an indicator of mitochondrial oxygen consumption and redox status[56], was significantly suppressed in both midzonal and pericentral areas and expression of P-selectin, an endothelial cell adhesion molecule[57], was depleted by this antagonist in the liver and the small intestine. It was also reported that excessive nitric oxide (NO) generation by hepatic cells, especially Kupffer cells, in response to LPS and inflammatory mediators, such as PAF, contributes to the pathogenesis of liver injury. In a model of rat liver-focused endotoxemia[58], pretreatment with specific PAF-R antagonists (BN-50739 or WEB-2170) reduced serum ALT and inducible nitric oxide synthase (iNOS) mRNA levels in the whole intact liver. In addition, pretreatment of cultured Kupffer cells with either of these antagonists resulted in significant inhibition of both LPS and PAF-induced iNOS mRNA and protein synthesis. Previous studies have shown that iNOS gene activation in macrophages by LPS depends on the transcription factor nuclear factor-kappa B (NF-κB), whose activation results in degradation of the α and β subunits of its inhibitor (IκB)[59]. It was shown that PAF-R antagonists attenuated the translocation of the active p65 subunit of NF-κB into the nucleus. A concomitant suppression of LPS-dependent degradation of the inhibitory protein subunit IκBα and intracellular calcium content of Kupffer cells was also noted. These results confirmed the protective effects of PAF antagonism against NO-mediated inflammatory liver tissue

injury during endotoxemia. In contrast to the results of the aforementioned studies, pretreatment of experimental animals with WEB-2086 one hour before intravenous LPS administration failed to inhibit LPS-induced sinusoidal neutrophil infiltration, evaluated by histological examination of hepatocellular necrosis and indicated by elevated plasma ALT activity, and did not significantly alter thrombocytopenia or plasma fibrinogen levels[60]. However, it has been speculated that the conflicting results might derive from variations related to the experimental models used in terms of the route of LPS administration, the degree of hepatocellular injury and temporal differences in the development of liver injury and histological evaluation. The role of PAF in alcoholic hepatitis in association with LPS administration has been studied by Murohisa *et al*[61]. Chronic ethanol administration remarkably sensitized experimental animals to the effects of LPS and severe hepatocellular injury associated with significantly elevated serum ALT, TNF-α and IL-8 levels was observed. Histological findings of liver injury included hepatocyte apoptosis and necrosis with marked neutrophil infiltration and enhanced Fas-receptor expression on hepatocytes and Fas-ligand expression on infiltrating neutrophils. Pretreatment with TCV-309 attenuated hepatocellular apoptosis and necrosis, neutrophil infiltration, Fas-receptor and Fas-ligand expression and decreased serum TNF-α levels. These protective effects suggested that PAF acts as a key mediator of endotoxin-induced liver injury in alcoholic hepatitis and possibly regulates Fas-receptor expression on hepatocytes via TNF-α, thus facilitating neutrophil accumulation and hepatocellular apoptosis. In an experimental model of liver-focused endotoxemia[62], LPS administration resulted in a significant increase in plasma-type PAF-AH mRNA and protein expression, particularly in Kupffer cells. A similar increase was noted in both circulating leukocytes and peritoneal macrophages as well as in all tissues examined. Administration of the PAF-R antagonists BN-50739 and WEB-2170 significantly inhibited the increase in PAF-AH activity in response to LPS, suggesting that PAF-R is probably involved in the upregulation of this enzyme, thereby minimizing the unfavorable hepatic and systemic effects of PAF-mediated injury. Two independent studies on D-galactosamine (D-GalN)/LPS-induced rat liver injury confirmed the protective effects of the potent PAF-R antagonist WEB-2086[63,64]. In the first study, a significant suppression of elevated transaminase levels and less severe histological changes were noted in the WEB-2086-treated animals compared to controls. Furthermore, PAF-R antagonism resulted in a significant attenuation of D-GalN/LPS-induced elevation of MDA and MPO levels and prevented the decrease of superoxide dismutase (SOD) in the liver tissue of WEB-2086-treated animals[63]. In the second study, both *in vivo* and *in vitro* effects of WEB-2086 were investigated[64]. The degree of hepatic injury and the levels of transaminases were significantly decreased in WEB-2086-treated animals after D-GalN/LPS administration and a significantly reduced number of infiltrating neutrophils was observed compared to the untreated group. *In vitro* evaluation revealed that the enhanced neutrophil adhesion to hepatic endothelial cells, either by stimulation with the sera collected from D-GalN/LPS treated animals or due to a direct effect of LPS, TNF-α or PAF, was also attenuated by WEB-2086 in a dose-dependent manner. These results strongly suggested a crucial role for PAF and PAF-R antagonists in this specific type of liver injury[63,64].

In an experimental study of acute, chemical-induced cholestasis by administration of the cholangiolitic hepatotoxicant alpha-naphthylisothiocyanate (ANIT), the role of PAF was investigated through pre- and post-treatment of study animals with WEB-2086[65]. ANIT-induced hepatotoxicity was confirmed by histological evaluation of periportal inflammation with marked neutrophil infiltration and biochemical parameters indicative of cholestasis. Treatment of the experimental group with WEB-2086 did not attenuate ANIT-induced liver injury as shown by increased plasma ALT and γ-glutamyl transferase (γGT) activities, or cholestasis, as indicated by increased plasma bilirubin concentration. These results suggested that soluble mediators other than PAF might be primarily involved in neutrophil-dependent liver injury caused by this hepatotoxic agent. In a most recent study evaluating the role of PAF in acetaminophen (APAP)-induced liver injury and regeneration[66], blood PAF levels and serum PAF-AH activity were measured in association with a biphasic pattern of APAP hepatotoxicity, as indicated by histological findings and three independent indices of liver regeneration. Blood levels of free PAF were significantly elevated during the first wave of liver injury and peak levels temporally coincided with the maximal levels of AST, ALT and ALP activity. Serum PAF-AH activity showed different time course from that of PAF and hepatic enzymes following the biphasic pattern of liver regeneration, with the first rise occurring before the elevation of PAF levels and the second rise coinciding with the second wave of regeneration, probably in response to the prior high levels of PAF. These results suggested that PAF plays a major role in the initial priming of the liver regeneration process, possibly acting as an intracellular mediator, and that PAF-AH seems to eliminate PAF-mediated liver injury mainly during the late phase of APAP-induced hepatotoxicity. The protective effects of the PAF-R antagonist BN-52021 on APAP-induced acute liver injury have been recently reported in a serial study conducted by Grypioti *et al*[67]. Intraperitoneal administration of this PAF-R antagonist resulted in a significant decrease of serum hepatic AST, ALT (indicative of necrosis) and alkaline phosphatase (ALP) (indicative of cholestasis) activities at several time points, accompanied by a remarkable elevation of serum PAF-AH activity at all time points compared to the control group. In addition, APAP-induced hepatocellular necrosis and apoptosis were significantly attenuated in BN-52021-treated animals. These results suggested that PAF might play a major role in liver injury and regeneration following acute APAP intoxication, underlying the beneficial effects of PAF-R antagonism.

In an experimental model of obstructive jaundice by bile duct ligation, a significant increase of liver tissue and plasma PAF levels was observed[68]. In another rat model of experimental obstructive jaundice of the same duration, PAF levels were significantly increased in liver tissue only, whereas no changes in plasma PAF levels were observed[69]. In this study, endotoxin was detected in portal

blood from jaundiced animals and endotoxin antagonism by neomycin plus polymyxin B significantly reduced local hepatic PAF levels. Moreover, isolated Kupffer cells from jaundiced rats demonstrated higher levels of cell-associated PAF compared to other liver cell types and spontaneous release of PAF by these resident hepatic macrophages was significantly increased compared to controls, supporting their putative role as the major source of hepatic PAF production. Pretreatment of isolated Kupffer cells with polymyxin B selectively prevented LPS-induced PAF production, implying that the interaction of Kupffer cells with portal endotoxin constitutes a potential mechanism that triggers local hepatic PAF synthesis. Treatment with PAF-R antagonists (BN-52021 or WEB-2170) partially prevented the increase in tissue levels of eicosanoids (TxB_2, 6-keto-PGF_{1a}), lipid peroxidation, as was prominent by reduced MDA production, and liver injury, evidenced by reduced plasma ALT levels. The effects of recombinant PAF-AH on tissue damage and antioxidant response have been recently evaluated in an experimental model of obstructive jaundice[70]. On the seventh postoperative day, AST, ALT, γGT, ALP, TNF-α and IL-6 levels were significantly lower in the PAF-AH treatment group compared to rats with obstructive jaundice which did not receive PAF-AH treatment, although still significantly higher compared to controls. Portal inflammation was also less severe and increased levels of antioxidant enzymes were noted in the treatment group, suggesting a protective effect of PAF-AH through PAF degradation.

Postoperative acute liver injury remains one of the most serious complications of extended hepatectomy and significantly influences postoperative morbidity and mortality. The implication of PAF in this type of injury was investigated in a rat model of 70% partial hepatectomy (PH)[71]. Post-PH, a significant rise in serum TNF-α, IL-6 and prostaglandin E_2 (PGE_2) levels was observed at 1, 3 and 6 h, respectively. No significant increase in portal venous endotoxin levels was noted. As early as 1 h post-PH, a significantly increased expression of PAF-R mRNA in Kupffer cells was noted in the group of hepatectomized rats compared to sham-operated ones. These data suggested that PAF possibly stimulated Kupffer cells leading to upregulation of PAF-R and induced acute liver injury via the release of TNF-α and IL-6 from these cells, which was subsequently suppressed by enhanced PGE_2 production. The role of PAF in LPS-induced liver injury with regenerating livers post-PH has also been investigated[72]. Treatment of experimental animals with TCV-309 before or after LPS administration resulted in significantly higher survival rates, effective suppression of plasma ALT and CINC levels and reduced liver tissue necrosis with infiltration of CINC-positive neutrophils, compared to the saline treated controls. Furthermore, attenuation of continuous CINC mRNA upregulation and NF-κB activation in the regenerating livers by TCV-309 confirmed the crucial role of PAF in LPS-induced liver injury, possibly via neutrophil accumulation and activation.

PAF and chronic liver injury

Liver cirrhosis is a terminal pathologic state induced by several biological and chemical agents capable of producing chronic liver injury. Irrespective of the exact mechanisms by which etiologic factors induce liver injury, they all trigger a common final pathway that leads to excessive fibrosis and nodular regeneration of the viable liver tissue[73,74]. PAF has been implicated as a mediator of the pathologic, metabolic and hemodynamic changes observed in cirrhosis both in humans and experimental animal models. Table 4 presents clinical and experimental studies on chronic liver injury in association with PAF synthesis and antagonism.

Table 4 PAF and chronic liver injury (cirrhosis)

Type of study	Method of cirrhosis induction	PAF levels	PAF-R antagonist used	Reference
Clinical				
	NA	↑	-	[76]
	NA	↓	-	[77]
	NA	NS	-	[81]
	NA	[1]	-	[82]
Experimental				
	CCl_4+ phenobarbital	↑	BN-52021	[84]
	TAA	[1]	-	[86]
	CCl_4	↑	-	[87]
	CCl_4	↑	BN-52021	[88]

[1] Not determined; NA = Not applicable; NS = Not significant; CCl_4 = Carbon tetrachloride; TAA = Thioacetamide.

In an early clinical report, increased PAF levels were observed in serum and ascitic fluid from decompensated cirrhotic patients, while compensated cirrhotics presented lower values but still higher than controls. In addition, serum PAF-AH activity was similar in all groups, supporting the conception of an enhanced production rather than a decreased degradation of PAF in cirrhotic patients[75]. Laffi *et al* further investigated alterations of PMN leukocyte function in nonalcoholic cirrhosis[76], based on well-established evidence of defective PMN function in patients with cirrhosis of alcoholic etiology[77]. PMN leukocytes from cirrhotic patients demonstrated *in vitro* a significantly reduced capacity to produce superoxide anion, determined by the reduction of cytochrome c, and a significant suppression of PAF synthesis, measured by [^{3}H]acetate incorporation into PAF and mass-spectrometric analysis, in response to physiological (opsonized zymosan) and nonphysiological (calcium ionophore, A23187) stimuli. Impaired PAF synthesis was attributed to defective PLA_2 activity implicated in the remodeling pathway. In addition, it was shown that most of synthesized PAF from stimulated PMNs remained cell-associated rather than being secreted, supporting the hypothesis that PAF may also function as an intracellular second messenger[78]. However, it has been argued that these *in vitro* results need to be confirmed in an *in vivo* model[79], since elevated serum PAF levels in cirrhotic patients have been previously reported. In a clinical study, patients with chronic cholestasis, especially those with end-stage primary or secondary biliary cirrhosis or cholangiocarcinoma, demonstrated increased serum PAF-AH activity[80]. Normalization of liver function after liver transplantation

was accompanied by a reduction to normal or near normal serum PAF-AH levels, suggesting that the liver plays an important role in the regulation of this degrading enzyme and, consequently, PAF. Moreover, it was shown that circulating levels of PAF are not a determinant of PAF-AH release from liver tissue. The crucial role of serum PAF-AH activity in the regulation of serum PAF levels has also been evaluated in patients with hyperbilirubinemic hepatobiliary disease[81]. In this clinical study, apart from an elevated serum PAF-AH activity in all groups, a significant correlation with serum cholesterol and low density lipoprotein (LDL) cholesterol levels was also noted in patients with liver cirrhosis and/or obstructive cholestasis. These findings confirmed the hypothesis that serum cholesterol, especially the LDL fraction, is a major factor influencing serum PAF-AH activity *in vivo*[82]. Although both PAF-AH activity/cholesterol and PAF-AH activity /LDL cholesterol ratios were elevated in all types of hyperbilirubinemic liver disease, the differential alteration of these ratios following normalization of bilirubin levels in each study group indicated that factors other than serum LDL levels, such as reduced biliary excretion of PAF-AH, might also be involved in the enhancement of PAF-AH activity.

Significantly higher blood PAF levels were observed in rats with experimental cirrhosis in a study evaluating the hemodynamic effects of PAF in cirrhosis[83]. Cirrhotic animals demonstrated a hyperdynamic circulatory status, similar to that induced by systemic intravenous PAF infusion[84], with increased cardiac output (CO) and decreased mean arterial pressure (MAP) and peripheral vascular resistance (PVR). In these animals, intravenous administration of BN-52021 resulted in a significant decrease in CO, attributed to the preexisting cardiac failure becoming apparent, along with a significant increase in PVR to near normal values, whereas MAP was only slightly elevated. These results strongly suggested a possible role for PAF in the regulation of hemodynamic changes in liver cirrhosis. The response of hepatic macrophages and the effects of PAF in experimental thioacetamide (TAA)-induced cirrhosis have been studied by Noda *et al*[85]. TAA administration resulted in an increase in hepatic macrophage population and cell size, followed by a marked increase in liver tissue hydroxyproline content, an index of collagen formation and deposition, indicative of cirrhosis. Intravenous PAF infusion during fibrosis induction before the development of cirrhotic changes significantly increased hepatic portal pressure and decreased oxygen consumption in TAA-treated animals compared to controls, possibly through elevation of the portal venous resistance by PAF-related molecules. The remarkable increase in prostaglandin D_2 (PGD_2) and thromboxane B_2 (TxB_2) levels in the perfusate induced by PAF was attributed to enhanced eicosanoid production and release by the increased population of active hepatic macrophages, suggesting a possible complex interaction between these components of chronic liver injury. Moreover, PAF-induced glycogenolysis was significantly lower in the TAA-treated group, indicating the disturbance of hepatocellular function in the early stages of cirrhosis as the likely cause of suppressed glucose production. Two serial studies on experimental carbon tetrachloride (CCl_4)-induced cirrhosis have further elucidated the role of PAF and its cognate receptor in the pathophysiology of liver cirrhosis. The first study revealed a pronounced increase in both basal and ET-1-stimulated PAF synthesis in Kupffer cells from cirrhotic rats compared to controls[86]. PAF binding capacity of these cells was also doubled, owing to increased density of functional PAF-Rs, although no significant difference in the PAF-R affinity was noted. It was also shown that Kupffer cells substantially contributed to increased hepatic and circulating PAF levels in cirrhosis. Consistent with the receptor binding data, the mRNA expression of PAF-R was also significantly increased. Since previous studies have shown that PAF stimulated eicosanoid synthesis in Kupffer cells, the enhanced production of prostaglandin E_2 (PGE_2), a hepatoprotective eicosanoid, observed in this study suggested an autocrine loop of PAF action which limited liver injury. The second study confirmed and extended these results in the hemodynamics of cirrhosis[87]. Compared to control rats, cirrhotic ones presented higher hepatic and arterial blood PAF levels, elevated PAF-R mRNA transcription and protein expression levels and higher baseline portal pressure. PAF infusion caused an augmented increase in portal pressure, whereas administration of BN-52021 resulted in a significant decrease of portal pressure in cirrhotic animals, without influencing systemic arterial hypotension. In addition, increased PAF-R density was observed in the contractile perisinusoidal stellate cells from cirrhotic livers. These results suggested that elevated circulating PAF levels, derived mainly from liver tissue, largely contribute to portal hypertension which is further exacerbated by upregulation of PAF-Rs in the cirrhotic liver.

CONCLUSIONS

During the past two decades, the role of PAF in a number of physiological and pathophysiological processes has been extensively investigated and documented. The characterization of PAF-Rs and their attendant signaling pathways, involving a complex interactive network of cytokines, second messengers and autacoids, has further elucidated the extracellular and intracellular actions of this potent proinflammatory lipid mediator. Liver pathology appears to be directly related to PAF synthesis and release by local or systemic cellular sources. The implication of PAF in virtually all types of liver injury and regeneration has been well-established by numerous experimental and clinical studies. It has been explicitly shown that PAF is a major contributor to the initiation and prolongation of liver IR injury, liver graft dysfunction and various types of acute liver injury, while its involvement in cases of chronic liver injury is possibly more complicated due to interference of several other mediators. However, there remain many incompletely characterized or probably unknown aspects in regard to the crucial role of PAF in the regulation and modification of cellular responses to various endogenous and exogenous biological or biochemical stimuli. The effects of potent and specific PAF-R antagonists in experimental conditions associated

with liver injury have provided emerging evidence for their potentially beneficial use in the clinical setting. The need to establish standardized experimental, or even clinical, models for the evaluation of the role of PAF and its antagonism is imperative in order to limit the variability of current models and provide an accurate means for reliable comparisons. Moreover, the present review could ideally serve as a comprehensive basis for future reference in this intriguing field of research.

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Combination of repeated single-session percutaneous ethanol injection and transarterial chemoembolisation compared to repeated single-session percutaneous ethanol injection in patients with non-resectable hepatocellular carcinoma

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Abstract

AIM: To evaluate the treatment effect of percutaneous ethanol injection (PEI) for patients with advanced, non-resectable HCC compared with combination of transarterial chemoembolisation (TACE) and repeated single-session PEI, repeated single-session PEI alone, repeated TACE alone, or best supportive care.

METHODS: All patients who received PEI treatment during the study period were included and stratified to one of the following treatment modalities according to physical status and tumor extent: combination of TACE and repeated single-session PEI, repeated single-session PEI alone, repeated TACE alone, or best supportive care. Prognostic value of clinical parameters including Okuda-classification, presence of portal vein thrombosis, presence of ascites, number of tumors, maximum tumor diameter, and serum cholinesterase (CHE), as well as Child-Pugh stage, α-fetoprotein (AFP), fever, incidence of complications were assessed and compared between the groups. Survival was determined using Kaplan-Meier and multivariate regression analyses.

RESULTS: The 1- and 3-year survival of all patients was 73% and 47%. In the subgroup analyses, the combination of TACE and PEI (1) was associated with a longer survival (1-, 3-, 5-year survival: 90%, 52%, and 43%) compared to PEI treatment alone (2) (1-, 3-, 5-year survival: 65%, 50%, and 37%). Secondary PEI after initial stratification to TACE (3) yielded comparable results (1-, 3-, 5-year survival: 91%, 40%, and 30%) while PEI after stratification to best supportive care (4) was associated with decreased survival (1-, 3-, 5-year survival: 50%, 23%, 12%). Apart from the chosen treatment modalities, predictors for better survival were tumor number (n < 5), tumor size (< 5 cm), no ascites before PEI, and stable serum cholinesterase after PEI (P < 0.05). The mortality within 2 wk after PEI was 2.8% (n = 3). There were 24 (8.9%) major complications after PEI including segmental liver infarction, focal liver necrosis, and liver abscess. All complications could be managed non-surgically.

CONCLUSION: Repeated single-session PEI is effective in patients with advanced HCC at an acceptable and manageable complication rate. Patients stratified to a combination of TACE and PEI can expect longer survival than those stratified to repeated PEI alone. Furthermore, patients with large or multiple tumors in good clinical status may also profit from a combination of TACE and reconsideration for secondary PEI.

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Key words: HCC; Single-session PEI; TACE; Survival; Prediction

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INTRODUCTION

The prognosis of untreated HCC remains poor. The 3-year survival ranges from 21% to 28% and declines to about 8% for advanced tumors[1]. The only therapies that currently offer potential cure are surgical resection and liver transplantation. Despite improved surgical techniques, only about 20% of HCC are amenable to resection, mainly due to advanced tumor growth and the underlying cirrhosis[2]. Systemic chemotherapy has shown no significant survival benefit in comparison to best supportive care[3]. Hence, locoregional modalities like transarterial chemoembolisation (TACE), percutaneous ethanol injection (PEI), laser induced thermo therapy (LITT), and radiofrequency ablation (RFA) are employed widely in non-resectable HCC in an attempt to prolong survival[4-7]. TACE is the preferred modality for patients with large and disseminated HCC in good physical status, whereas repeated PEI has shown efficacy in cases of small tumors[8]. Both modalities have proven their effectiveness in terms of tumor response and survival, even more so when combined[9-17].

PEI has gained importance in small HCC due to its safety and ease of operation. The method is both effective and economically sound. The survival data of patients with small tumors after PEI are comparable to those after surgical resection with 5-year survival rates between 32%-59%[15, 18-22]. The amount of ethanol applicable during one session was initially limited. With the introduction of the single-session high-dose technique under general anaesthesia by Livraghi and co-workers, the indications for PEI have been extended[23]. The injection of higher quantities of ethanol during one session under mechanical ventilation offers the advantage of treating larger tumors and more lesions at a time, requiring fewer interventions. Furthermore, tumors in difficult accessible areas (e.g. liver segments 8, 1 or 2) can be better accessed in deep inspiration under mechanical ventilation.

According to physical status and tumor extent patients with non-resectable HCC were stratified in an interdisciplinary conference to repeated single-session PEI, repeated TACE sessions, a combination of TACE and repeated single-session PEI or to best supportive care[24]. Aim of this prospective study was to evaluate all patients of our institution who received PEI treatment from 1999 to 2003. Using multivariate regression analyses of pre-treatment variables, survival predictors were identified. In subgroup analyses, patients stratified to repeated PEI alone were compared to those stratified to a combination of TACE and PEI. Furthermore, long-term results of those patients who were reevaluated and switched secondarily to PEI treatment after being initially stratified to repeated TACE and of those patients who received PEI after being initially stratified to best supportive care for advanced disease were analysed[9].

MATERIALS AND METHODS

Patients

One hundred and one patients (age range 35-88 years, 85% male, mean observation time 26 ± 17 mo) with non-resectable histologically-confirmed HCC who received PEI treatment from July 1999 until July 2003 in our institution were included into the present study.

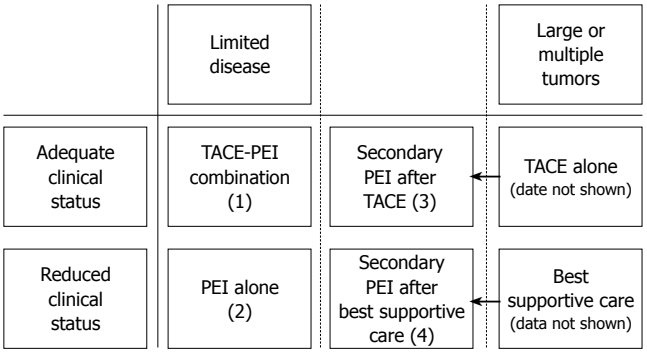


Figure 1 Stratification algorithm for the treatment of advanced unresectable HCC.

Stratification

In an interdisciplinary gastroenterological-radiological conference, patients were stratified to one of the following treatment modalities according to physical status and tumor extent (Figure 1): combination of TACE and repeated single-session PEI, repeated single-session PEI alone, repeated TACE alone, or best supportive care. After thorough explanation of the procedures, informed consent was obtained from each patient prior to therapy. The data of the TACE-PEI combination group (1) and of the PEI alone (2) group were analyzed. In addition, patients with extensive disease (large or multiple tumors) were reconsidered and in some cases restratified to secondary PEI treatment (Figure 1). These patients were evaluated in two separate subgroups: patients switched secondarily to PEI after being initially stratified to repeated TACE (3) or after being initially stratified to best supportive care (4).

Exclusion criteria

Contraindications against regional treatment were extrahepatic tumor manifestation, sepsis, hepatic encephalopathia, elevated serum ammonia > 47 μmol/L, platelet counts < 50 000/μL, prothrombin activity < 50%, serum creatinine >120 μmol/L, left ventricular ejection fraction < 50%, heart attack within the previous 12 mo, heart insufficiency >NYHA stage II. The inclusion into the liver transplantation program was not a contraindication.

Further contraindications against PEI were significant perihepatic ascites or inadequate parameters for general anaesthesia.

Further contraindications against TACE were portal vein thrombosis, impossible catheterization of the hepatic artery, cholinesterase (CHE) < 2 mU/mL (21℃), inadequate functional, hematological or biochemical parameters for chemotherapy.

Outcome assessment

Pre- and post-treatment evaluation included physical examinations, the assessment of clinical parameters, toxicities, adverse events and survival and the evaluation of tumor markers as well as hematological and biochemical parameters. To classify cardiac function and to detect right heart hypertrophism and pulmonary hypertension,

echocardiography, cardiac scintigraphy, and ECG were performed.

PEI treatment

Single-session PEI therapy was performed during general anaesthesia under realtime ultrasound guidance. Before each session the patients underwent a complete abdominal ultrasound examination. After thorough skin disinfection, absolute sterile ethanol (96%) was injected transcutaneously into the tumor using a 0.7 mm needle (Angiomed, Karlsruhe, Germany) and a biopsy-transducer (3.5 MHz, Toshiba, Neuss, Germany). Starting at the most distal part of the tumor, ethanol was continously injected while carefully retracting the needle until the tumor echogenicity became homogenous. After complete ethanol administration, short-term follow-up ultrasound control examinations were performed. Intravenous infusions of antibiotics and of fluid replacement were given before and for two days after PEI.

Six weeks after therapy, doppler ultrasound control examinations were performed. In case of detectable remaining tumor vascularisation (indicating remaining vital tumor tissue), tumor growth or new lesions, further PEI treatment was initiated. In case of uncertain vascularisation echocontrast enhanced ultrasound and a biphasic CT or contrast-enhanced MRI scan were performed. In case of no detectable tumor vascularization, patients were followed up every three months.

TACE treatment

TACE was performed under local anaesthesia. Before each TACE session, a biphasic CT scan of the abdomen was performed to show tumor extent, and to check for possible extrahepatic disease. After aortography and a selective mesentericoportography to verify vascular anatomy and the patency of the portal vein, a selective hepaticography was performed using a standard diagnostic catheter or, if necessary, a coaxial system. According to tumor vascularisation and distribution a mixture of cisplatin (50 mg/m²), doxorubicin (50 mg/m²), 450 to 900 mg degradable starch microspheres (Spherex, Pharmacia, Erlangen, Germany) and 5 to 30 mL iodized oil (Lipiodol© Ultra-Fluide, Guerbet, Sulzbach/Ts., Germany) were administered under fluoroscopic control. In case of stasis or reflux, the injection was stopped until the arterial flow resumed. After complete administration, a control hepaticography was performed to document the arterial perfusion and the reduction of tumor vascularisation. Intraarterial analgesia (Dolantin©, Pethidin, Aventis, Bad Soden/Ts., Germany) was sufficient to control local pain in all cases. Intravenous infusions of antibiotics and fluid replacement were given before therapy and for two days after TACE, accordingly.

Statistical analysis

Data analysis was performed employing the Statistical Package for the Social Sciences Version 12.0 for Windows (SPSS© Inc., Chicago IL, USA). For comparison of distributions the Wilcoxon-test was used, for metric variables the chi-square test and the Fisher's exact test for nominal variables. Results were presented as mean ± standard deviation or frequencies [*n* (%)].

Overall survival of all patients and survival of the subgroups were determined by Kaplan-Meier analyses. Kaplan-Meier curves were statistically compared by the log-rank-test. In order to quantify the influence of the treatment strategies on the survival outcome, a multivariate logistic regression analysis (enter method) was carried out. The clinical parameters that differed between the four treatment groups at baseline (Okuda-classification, presence of portal vein thrombosis, presence of ascites, number of tumors, maximum tumor diameter, and CHE) were entered into the regression model as well as Child-Pugh stage, α-fetoprotein (AFP), fever, incidence of complications, and the type of treatment administered. The model rendered the relevance of the variables tested competitively (R-square-value) and identified significant predictors for survival.

Subsequently, Kaplan-Meier curves were computed for all patients stratified separately for every significant predictor of the regression analysis in order to demonstrate the influence of the predictors on survival probabilities.

A P-value < 0.05 was considered significant, and a P-value < 0.001 was considered highly significant.

RESULTS

Disease severity at outset

The patient set included a high proportion of patients with advanced stages of liver cirrhosis and tumor extent. Forty-one percent of the 101 patients presented with cirrhosis of Child-Pugh stage B, 15% with stage C. Okuda-scores II and III were present in 62%. Portal vein thrombosis was found in 18% and ascites requiring intensified diuretic therapy in 42% of the patients. In only 14% uninodular tumors were detected, in 70% up to 5 tumor lesions and in 15% multiple tumors. The mean maximum tumor size was 53 (± 27) mm in diameter. High mean AFP levels of 1188 ng/mL (± 6802) were seen, and the mean CHE levels were reduced to 3.3 mU/mL (± 1.6). The hepatitis B status was positive in 27% of the patients, and hepatitis C status in 22%.

The baseline characteristics of the four subgroups differed due to the selection criteria employed. A comparison of the frequencies and mean values of clinical variables is shown in Table 1.

Significant deviations of the subgroups were shown for the Okuda score, the frequencies of portal vein thrombosis and ascites, the number of tumor lesions, the maximum size of tumor diameter and the liver parameters CHE, bilirubin, and AST.

Treatment

Overall, 268 PEI-interventions were performed in 101 patients (2.7 ± 1.7 sessions per patient). The mean injected ethanol volume was 42 mL (3-117) per session. The mean hospitalisation time was 6.8 ± 4.8 d. In 43 patients (40.5%) intensified diuretic therapy was needed to reduce pre-treatment ascites. According to the stratification algorithm 37 patients received combination treatment of TACE and repeated PEI (1) and 34 patients received PEI alone (2). Ten patients primarily stratified to repeated TACE treatment

Table 1 Baseline patient characteristics of the four evaluated treatment subgroups

		PEI alone (2) *n* = 34	Sec PEI after best supp. care (4) *n* = 20	TACE-PEI combinat. (1) *n* = 37	Sec PEI, after TACE (3) *n* = 10	*P*-value
Child-Pugh	A	14 (41%)[1]	7 (35%)	18 (49%)	6 (60%)	
	B	14 (41%)	10 (50%)	19 (51%)	4 (40%)	
	C	6 (18%)	3 (15%)	0 (0%)	0 (0%)	
Okuda	1	12 (36%)	2 (10%)	16 (46%)	6 (67%)	0.05
	2	20 (61%)	14 (74%)	16 (46%)	3 (33%)	
	3	1 (3%)	3 (16%)	3 (8%)	0 (0%)	
Gender	male	27 (80%)	19 (95%)	33 (89%)	7 (70%)	NS
Portal vein thromb	yes	1 (3%)	7 (35%)	9 (26%)	1 (10%)	0.02
Ascites	yes	12 (35%)	13 (65%)	16 (44%)	1 (10%)	0.03
No of tumors	1	4 (12%)	3 (15%)	5 (13%)	2 (20%)	0
	2	8 (23%)	3 (15%)	4 (11%)	1 (10%)	
	3	13 (38%)	1 (5%)	2 (6%)	2 (20%)	
	4	7 (21%)	0 (0%)	9 (24%)	0 (0%)	
	5	2 (6%)	1 (5%)	17 (46%)	1 (10%)	
	multiple	0 (0%)	12 (60%)	0 (0%)	4 (40%)	
Tumor size	mm	41 (14)[2]	68 (39)	52 (20)	75 (32)	0
No of PEI	n	2.3 (1.6)	3 (2.8)	2.6 (1.2)	3.3 (2.3)	NS
Weight	kg	75 (12)	75 (10)	77 (16)	78 (18)	NS
AFP	ng/mL	235 (515)	5743 (15910)	257 (476)	28 (44)	NS
CRP	mg/L	65 (61)	92 (68)	97 (73)	83 (58)	NS
Bilirubin	μmol/L	35 (24)	36 (36)	23 (21)	13 (7)	0.008
CHE	mU/mL	2.8 (1.6)	2.6 (1.4)	3.7 (1.4)	4.6 (1.1)	0.005
LDH	U/L	226 (77)	218 (41)	218 (58)	198 (27)	NS
Protein	g/L	73 (7)	74 (8)	73 (6)	71 (4)	NS
AST	U/L	43 (45)	39 (26)	37 (41)	15 (6)	0.01
ALT	U/L	37 (34)	33 (24)	35 (31)	14 (5)	NS
AP	U/L	204 (98)	254 (125)	212 (108)	320 (412)	NS
G-GT	U/L	105 (75)	107 (75)	153 (172)	123 (203)	NS
Leukocytes	1000/μL	5.6 (2.2)	5.5 (1.4)	6.0 (2.5)	6.3 (1.8)	NS
QUICK	%	75 (18)	79 (13)	85 (18)	88 (21)	NS
Thrombocytes	1000/μL	120 (56)	145 (76)	124 (62)	213 (115)	NS
Creatinine	μmol/L	82 (38)	106 (120)	78 (35)	72 (17)	NS

NS: Not significant, [1] *n* (%), [2] Mean (standard deviation). TACE-PEI combination, PEI alone, secondary PEI after TACE discontinuation, and secondary PEI after best supportive care are displayed. The *P*-values for comparison of baseline characteristics between these subgroups are shown. CHE: Cholinesterase, AP: Alkaline phosphatase, G-GT: Gamma glutamyl transferase.

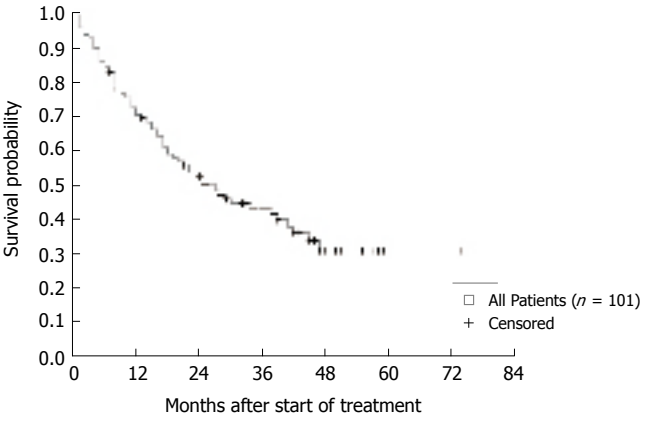


Figure 2 Kaplan-Meier curve showing the survival probabilities of all 101 patients.

only, were switched after TACE discontinuation to secondary PEI treatment (3). Another 20 patients who were primarily stratified to best supportive care, were considered treatable by PEI and received subsequently repeated PEI sessions in order to decrease tumor load in individual therapy settings (4).

Reasons for discontinuation of TACE in the patients continued secondarily on repeated PEI sessions were adverse events ($n = 6$), efficacy failure/progressive disease ($n = 3$) or achievement of preconditions for PEI treatment due to tumor regression ($n = 1$). In those patients primarily stratified to best supportive care secondary PEI treatment was performed because of improvement of general health status ($n = 9$), of patient´s request ($n = 7$), of reduction of ascites ($n = 2$), or as a bridge to liver transplantation ($n = 2$).

Seven of the 101 patients received liver transplants during follow-up (7%). Two of these patients had been stratified to combination treatment of TACE and PEI (1), four patients to repeated PEI alone (2), and one patient secondarily to repeated PEI after primary stratification to TACE (3).

Survival

The overall 1- and 3-year-survival rates of the 101 patients were 73% and 47%, respectively. Kaplan-Meier analyses of all patients rendered survival probabilities of 72% after 1 year, 43% after 3 years, and 32% after 5 years (Figure 2).

A comparison of the Kaplan-Meier survival curves for the four subgroups is shown in Figure 3. Survival

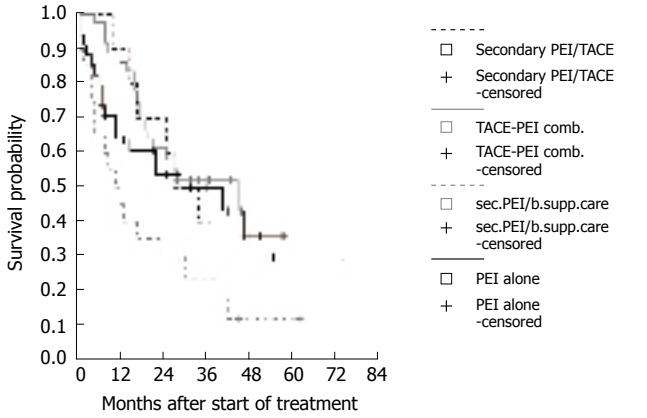


Figure 3 Comparison of Kaplan-Meier curves showing the survival probabilities of the four subgroups: TACE-PEI combination, PEI alone, secondary PEI after TACE, secondary PEI after best supportive care. The highest survival probabilities can be expected in the combination treatment group initially stratified to TACE followed by repeated single session PEI.

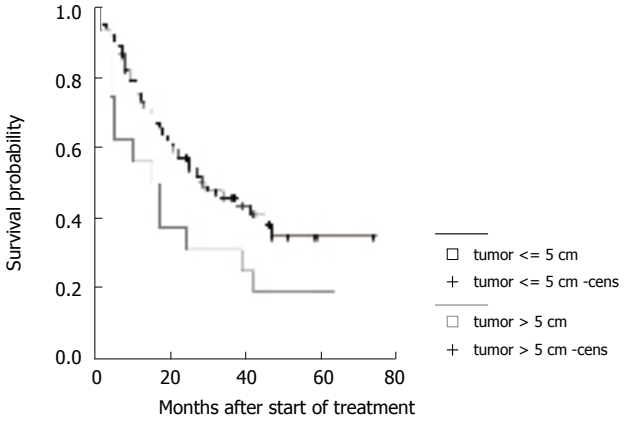


Figure 4 Comparison of the Kaplan-Meier curves for the parameter maximum tumor diameter (5 cm or less *vs* more than 5 cm) prior to stratification. The survival probabilities of the subgroup with smaller tumors are significantly higher (logrank: $P = 0.02$).

Table 2 Multivariate regression model of predictors for longterm survival of three years and more after start of therapy

Predictors	Cut-off	*P*-value	Odd's ratios	Confidence interval
AFP	< 400 ng/mL	NS		
Fever	< 38℃	NS		
CHE-level at follow up	Change < 1 mU/mL	0.05	6	2.4-10.1
No of tumor lesions	⩽ 5	0.01	11.1	3.3-22.3
Child-Pugh	Child A	NS		
Ascites	No ascites	0.03	12.2	4.7-30.3
Portal vein thrombosis	No thrombosis	NS		
Okuda	Okuda stage 1	NS		
Severe complications	No complication	NS		
Maximum tumor size	⩽ 5 cm	0.04	6.1	1.3-17.5
Type of treatment	TACE-PEI comb (I) *vs* other groups	0.05	4.3	1.2-14.7

P-values, Odd's ratios and confidence intervalls are shown for all significant variables. $R^2 = 43\%$.

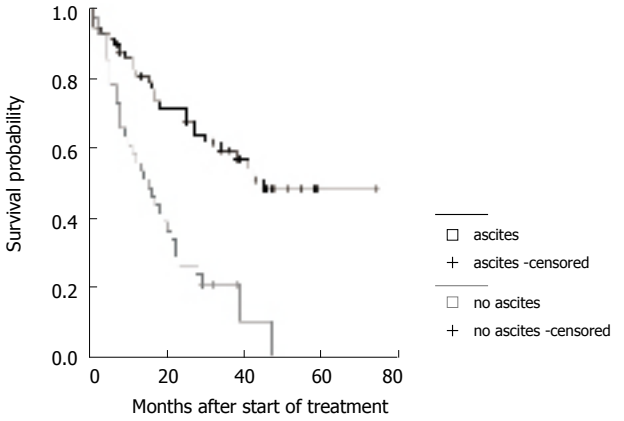


Figure 5 Comparison of the Kaplan-Meier curves for the parameter ascites prior to stratification (ascites *vs* no ascites). Significantly higher survival probabilities can be expected for the subgroup without ascites (logrank: $P < 0.001$).

probabilities differed between the subgroups. Significant differences were detected by log rank-tests comparing secondary PEI after best supportive care (4) to combination treatment of TACE and PEI (1) ($P < 0.001$) and to PEI treatment alone (2) ($P < 0.05$). There was a trend towards better survival in the combination group (1) compared to PEI alone (2) ($P = 0.1$).

The 1-, 3-, and 5-year survival probabilities of the subgroups were 90%, 52%, 43% for the combination group (1); 65%, 50%, 37% for the PEI alone group (2); 91%, 40%, 30% for the secondary PEI group after TACE discontinuation (3); and 50%, 23%, 12% for the secondary PEI group after best supportive care stratification (4).

Predictors of three-year-survival

From all clinical baseline characteristics that differed between the four subgroups at the outset of the study, pre-treatment ascites, the number of tumor lesions, the maximum tumor size, and CHE were significant predictors in the multivariate enter-model (Table 2). Additionally, the type of treatment administered proved to have independent predictive value in the model.

The probability to survive longer than three years was 6-fold higher in patients with stable CHE levels under therapy and in patients with tumors of less than 5 cm in maximum diameter, 11-fold higher in patients with five tumor lesions or less and 12-fold higher in patients without ascites prior to therapy. Treatment with TACE and PEI combination (1) led to a 4-fold higher survival probability compared to the other subgroups (2-4) independently of the clinical parameters tested in the model.

Stratification of predictors in Kaplan-Meier analyses

Survival curves were generated for all significant parameters of the prediction model and stratified according to the cut-offs employed (Figure 4, Figure 5, Figure 6 and Figure 7). The differences of the stratified subgroups were significant in all parameters shown (logrank-test: $P < 0.05$).

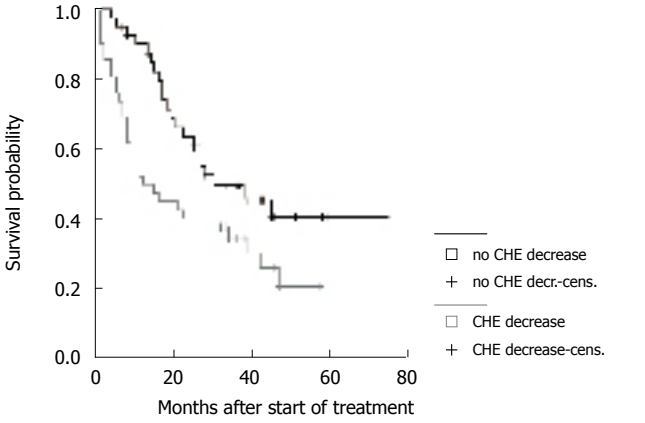


Figure 6 Comparison of the Kaplan-Meier curves for the parameter decrease in serum CHE-levels after PEI (decrease >1 mU/mL *vs* no decrease). The subgroup with stable CHE levels can expect significantly better survival probabilities (logrank: $P = 0.02$).

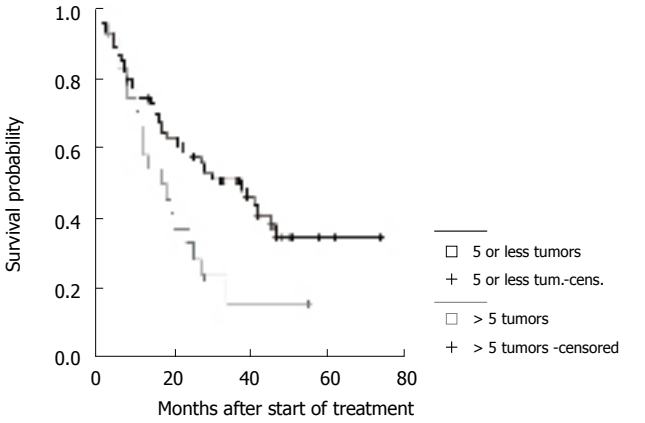


Figure 7 Comparison of the Kaplan-Meier curves for the parameter number of tumor lesions (5 or less tumors *vs* more than 5 tumors). The survival probabilities of the group with 5 or less tumors are significantly higher (logrank: $P < 0.05$).

The survival was improved significantly in patients with stable CHE-levels under therapy as a parameter of the liver function, in patients without ascites prior to therapy, in patients with a maximum tumor diameter of less than 5 cm, and in patients with five or less tumor lesions.

TACE complications

A total of 67 TACE procedures were performed. After 7 (10.4%) procedures complications were recorded. There were two cases of reversible leukopenia, one case of reversible pancytopenia, two cases of dissection of the hepatic artery, one case of reversible liver failure, and one inguinal hematoma.

PEI complications

Sixty-nine (25.7%) complications occurred after the 268 PEI sessions performed. Forty-five of these (16.8%) were considered minor [40 ascites (29 discrete, 7 intermediate, 4 severe), 3 pleural effusion, 1 oedema of the lower leg]. In 24 interventions (8.9%) major complications were observed (5 segmental liver infarctions, 2 focal liver necroses, 4 liver abscesses, 1 pulmonary embolism, 5 bleedings into liver or abdomen, 3 thromboses of the portal vein, 1 thrombosis of the superior mesenteric vein, 1 pancreatitis, 1 cholecystitis, and 1 cholangitis). All complications were managed non-surgically, and one liver abscess needed local drainage. There were no instances of needle-track seeding.

The mortality within 2 wk after PEI was 2.8% (3 patients). Two patients died with the signs of an acute right heart decompensation (one patient suffering from pre-existing alcoholic congestive heart disease with pulmonary hypertension, the other suffering from previously unknown right ventricular hypertrophy). The third patient developed a hypertensive crisis followed by an apoplectic insult.

DISCUSSION

In patients with HCC, survival is influenced by the etiology and characteristics of the tumors as well as the degree of cirrhosis and the remaining hepatic function. Since the degree of cirrhosis is rarely affectable in most cases, improval of survival can be expected mainly through effective tumor treatment. To date, there is still no standard treatment for advanced, unresectable HCC. The lack of efficient systemic chemotherapy against HCC and the non-resectability in most cases led to the development of various locoregional treatment modalities[22]. Increased anti-tumor efficacy might be achieved through a combination of different modalities carefully tailored to each patient.

PEI was introduced in 1983 and has become a standard treatment for small HCC[25]. The rationale for PEI is complete tumor necrosis resulting from cellular dehydration, protein denaturation, and chemical occlusion of tumor-feeding vessels. The method is best applicable in those HCCs that are surrounded by firm liver cirrhosis reducing the washout of ethanol. Ultrasound guidance allows for realtime monitoring of ethanol injection and distribution. Although regular PEI can be performed under local anaesthesia, general anaesthesia and endotracheal intubation have greatly facilitated the implementation of the single-session technique in terms of reduced pain and patient movement, thus extending the range of treatable tumors[23]. The major draw-back of PEI is a comparatively high recurrence rate of more than 30%[14]. Plausible reasons are inhomogeneous ethanol distribution within the tumor and the limited effect on extracapsular tumor spread. About 30% of small HCC (< 3 cm) have already developed invasive growth leading to microscopic intrahepatic metastases not detectable by current imaging[12]. The effect of PEI may be incomplete even in small tumors[26].

It is postulated that while the visible tumors are ablated by PEI, microscopic metastases are effectively destroyed by TACE. Furthermore, TACE is supposed to break up intratumoral septae and to create a fibrous wall around the hypervascularized tumors thus leading to a more homogenous distribution of ethanol during subsequent PEI[27,28]. Hence, the combination of TACE and repeated PEI, introduced in 1991, has shown superior results

compared to PEI or TACE alone[11-14,29]. This concept was also adopted by our institution as described previously[24]. After careful interdisciplinary evaluation patients with non-resectable HCC were treated by a combination of TACE and repeated PEI, if possible. Those patients not amenable to combination treatment were stratified to either modality alone or to best supportive care according to their individual situation. Furthermore, patients with large or multiple tumors were reevaluated under therapy and secondarily switched to PEI when considered possible (Figure 1).

In the present prospective study there was a comparatively high proportion of patients with decreased liver synthesis Child-Pugh B (41%) and C (15%) and portal vein thromboses (18%). Only 14% of the patients had single tumors while 15% had multiple disease and the mean tumor size was 53 mm (Table 1). Because both liver function and tumor extent affected therapy decisions, the present study was not a controlled trial but a prospective therapy evaluation based on a stratification scheme according to individual patient characteristics. The results were therefore not unexpected: patients stratified to PEI-therapy alone had worse pre-treatment conditions in terms of clinical status and liver function compared to those stratified to the combination of TACE and PEI (Table 1).

Overall observed 1- and 3-year-survival of all 101 patients was 72% and 47%. Kaplan-Meier analyses revealed a 1, 3, and 5 year survival probability of 90%, 52%, and 43% after initial stratification to TACE followed by PEI and of 65%, 50%, and 37% after PEI alone (Figure 3). Superior survival of combining TACE and PEI compared to either modality alone has been proven by several investigators. Bartolozzi *et al* detected superior recurrence-free survival of patients after combination of TACE and PEI (100% and 72% after 1 and 3 years), compared to repeated TACE alone[13]. Allgaier *et al* found a survival benefit of patients stratified to combination of TACE and PEI (*vs* PEI alone) due to a lower recurrence rate [11]. Koda *et al* showed superior survival after 1, 3, and 5 years (100%, 80%, and 40%) for patients after combination treatment (*vs* PEI alone), and Kamada *et al* recorded 1-, 3-, and 5-year survival rates of 90%, 65%, and 50% after combination treatment[12,14].

Our results are hence in line with the above data; however, there are differences in terms of patient criteria. The former studies did apply comparatively strict inclusion criteria in terms of tumor number and size. It should be noted that the patients consecutively included into the present study were in more advanced stages of both tumor extent and liver cirrhosis. Being a liver transplantation center in Northern Germany, advanced stages of tumor extent and cirrhosis were included in an attempt to prolong survival of those patients awaiting possible liver transplantation: patients with multinodular disease up to 5 tumors with less than 5 cm diameter or singular tumors up to 7 cm in diameter were primarily stratified to combination treatment. In this setting, the present survival data were higher than expected. The overall survival of our patients after combination therapy is comparable to reported survival data of tumor-free patients with liver cirrhosis[30]. Consequently, the present combination treatment of HCC can be considered effective for our patients with advanced disease.

The 10 patients with large (>7 cm) or multiple ($n > 5$) tumors who were switched from repeated TACE treatment to secondary PEI after re-evaluation (Figure 1), were indeed amenable to secondary PEI after receiving initial TACE as stated by other investigators[31]. Interestingly, 1-, 3-, and 5-year survival of this group (91%, 40%, and 30%) was comparable to those patients with limited disease who were primarily stratified to combination treatment (90%, 52%, and 43%) (Figure 3). From our experience it may be advisable to reconsider patients for PEI even after repeated TACE treatment, especially if TACE therapy cannot be continued.

The 1-, 3-, and 5-year-survival of the 20 patients with severely impaired liver function and advanced tumors, who were switched to repeated PEI after initially being stratified to best supportive care, was comparatively poor (50%, 23%, and 12%). However, it was superior to a large set of patients treated by best supportive care only or systemic therapy from our institution (1- and 3-year-survival: 32% and 2%)[24]. Large tumors can technically be treated by PEI as a salvage therapy, however, at the increasing risk of systemic, toxic side-effects and other severe complications as previously suggested[9]. From our experience we would not generally recommend single-session PEI as a salvage therapy in patients with both disseminated disease and severely impaired liver function who cannot receive TACE as initial treatment. Still, there may be patients in this group who might profit. It seems as if sufficient clinical status and/or the effect of initial TACE before PEI is needed to enhance anti-tumoral activity and survival.

In the multivariate analyses the parameters of limited tumor size and number, no ascites prior to therapy, and stable levels of CHE under therapy were associated with better survival. The type of treatment administered seemed to have independent predictive value, however, the significance of this result may be reduced due to the limited patient numbers (Table 2). Although screening programs improved the early detection of HCC, large non-resectable tumors are still frequently encountered and remain a major therapeutic challenge[32]. As expected, tumor size did affect survival. This might be explained by the fact that small tumors tend to have lower gradings than large tumors. Histological studies revealed that tumors up to 3 cm diameter are mostly well differentiated while large tumors are in general less differentiated[18,33,34]. Furthermore, there is a greater risk in larger HCC of peripheral satellite tumors that escape pre-treatment staging[35]. The functional status of the liver has been known to be a major predictor in PEI treatment as it is reflected in the significance of pre-treatment ascites and CHE-levels under therapy in the present study[19]. Both TACE and PEI might further impair the remaining liver function. The regeneration of functional liver parenchyma is possibly delayed as indicated by decreased liver synthesis parameters after treatment (data not shown).

While regular PEI is considered a low-risk procedure, severe complications, including death have been reported[36-38]. In the present study on repeated single-session PEI in advanced tumors the overall complication

rate was 25.7% (n = 69) including 8.9% (n = 24) major complications, however, all could be managed non-surgically. We consider these instances acceptable because single session PEI represents a more aggressive approach than regular PEI[37]. Giorgio *et al* stated a mortality of 1.8% after single-session PEI[31]. There were also three deaths (2.8%) within 14 days after PEI among our patients. Two patients died with the signs of right ventricular decompensation, one patient with a hypertensive crisis followed by an apoplectic insult. Although there was no direct link to PEI, a connection seems possible, as other authors stated cases of pulmonary hypertension after PEI were possibly due to shunting[23]. We did not observe any instance of needle track seeding, as described by others[21].

In conclusion, in our set of patients with advanced non-resectable HCC the combination of TACE and repeated single-session PEI is effective and shows superior results compared to repeated single-session PEI alone. Even for patients with large (>7 cm) or multiple (n > 5) tumors initially stratified to repeated TACE treatment alone PEI treatment is effective in terms of survival at an acceptable complication rate. The predictors including limited tumor size and number, no pre-treatment ascites, and stable levels of CHE under therapy are independently associated with better survival. Our experience further suggests that in advanced disease carefully tailored combination therapy is more advantageous than single therapy. The optimal strategy against advanced HCC, however, has still to be determined by future trials.

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LIVER CANCER

Activation and dramatically increased cytolytic activity of tumor specific T lymphocytes after radio-frequency ablation in patients with hepatocellular carcinoma and colorectal liver metastases

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Abstract

AIM: To assess if a specific cytotoxic T cell response can be induced in patients with malignant liver tumors treated with radio-frequency ablation (RFA).

METHODS: Six Patients with liver metastases of colorectal cancer and 6 with hepatocellular carcinoma (HCC) underwent RFA. Blood was sampled before, 4 and 8 wk after RFA. Test antigens were autologous liver and tumor lysate obtained from each patient by biopsy. Peripheral T cell activation was assessed by an interferon gamma (IFNγ) secretion assay and flow cytometry. T cells were double-stained for CD4/CD8 and IFNγ to detect cytotoxic T cells. The ratio of IFNγ positive and IFNγ negative T cells was determined as the stimulation index (SI). To assess cytolytic activity, T cells were co-incubated with human CaCo colorectal cancer and HepG2 HCC cells and release of cytosolic adenylate kinase was measured by a luciferase assay.

RESULTS: Before RFA SI was 0.021 (± 0.006) for $CD4^+$ and 0.022 (± 0.004) for $CD8^+$ T cells against nonmalignant liver tissue and 0.018 (± 0.005) for $CD4^+$ and 0.021 (± 0.004) for $CD8^+$ cells against autologous tumor tissue. Four weeks after RFA SI against tumor tissue increased to 0.109 (± 0.005) for $CD4^+$ and 0.11 (± 0.012) for $CD8^+$ T cells against HCC, and to 0.115 (± 0.031) for $CD4^+$ and 0.15 (± 0.02) for $CD8^+$ cells for colorectal metastases ($P < 0.0001$). No increased SI was observed with nonmalignant tumor tissue at all time points. Before RFA cytolytic activity against the respective cancer cells was low with 2.62 (± 0.37) relative luminescence units (RLU), but rose more than 100 fold 4 and 8 wk after RFA. Spontaneous release was < 2% of maximum release in all experiments.

CONCLUSION: Patients with primary and secondary tumors of the liver show a significant tumor-specific cytotoxic T-cell stimulation with a dramatically increased tumor specific cytolytic activity of $CD8^+$ T cells after RFA.

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Key words: CD4; CD8; Cytotoxic; Immune response; Immunology; Immunotherapy; Interferon gamma; T cells; NK; Therapeutic vaccination

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INTRODUCTION

Open surgery is still the gold standard for the treatment of hepatocellular carcinoma (HCC) and liver metastases of colorectal cancer. However, more than 75% of these patients are non-resectable for various reasons, such as size and location of the tumor. Radio-frequency thermal ablation (RFA) is a common therapy option for unresectable liver tumors. Thus it was shown that RFA and laser-induced thermo-therapy (LiTT) can achieve survival times comparable to surgery in selected patients[1-3]. RFA uses a radio-frequency current to induce coagulation necrosis which results in a local thermal necrosis accompanied by

local inflammation around the necrotic center and by a hemorrhagic rim with lymphoplasmacellular infiltrates[4]. This rim is supposed to be a hot spot for recruitment and activation of tumor-specific T lymphocytes. By using the VX2 hepatoma model in rabbits we could show that RFA can induce a strong mononuclear infiltration around the implanted tumor. It remained unclear if the observed T cell response was merely directed to papilloma viral antigens that are derived from the VX2 hepatoma compared to a tumorspecific T-cell reaction. However, we could recently demonstrate a marked tumor-specific peripherial T cell response in RFA-treated *vs* untreated rabbits with the VX2 hepatoma[5].

The aim of the present study was to demonstrate if an antitumor T cell response is also inducible in patients who are subjected to RFA. Therefore, we studied the cytotoxic ($CD8^+$ and $IFN\gamma^+$) T cell and T-helper cell ($CD4^+$ and $IFN\gamma^+$) response of peripheral blood mononuclear cells (PBMC) against autologous nontumoral and tumoral liver tissue in patients with primary and secondary neoplasms of the liver. In addition, we measured the cytolytic activity of the $CD8^+$ and $IFN\gamma^+$ T cells against CaCo colorectal cancer and HepG2 HCC cells.

MATERIAL AND METHODS

The study was approved by the ethical committee of the University of Erlangen-Nuremberg and performed according to the declaration of Helsinki. All treated hepatocellular carcinomas and colorectal cancer metastases were confirmed histologically prior to therapy. All patients enrolled into the study signed an informed consent prior to inclusion.

Patients

In this preliminary group patients with up to 3 tumor nodules within the liver, with a maximum diameter of 6 cm per lesion, were enrolled in the study. Prior local ablative therapy (LiTT, RFA, ethanol injection) or prior chemoembolisation of the malignant liver tumor was an exclusion criterion. Additionally, a curative option by resection or liver transplantation was ruled out. Patients with at least one of the following findings were also excluded: Karnofsky index < 60, thrombocytes < 50 000μL, prothrombin activity < 50%, partitial thromboplastin time > 80 s. No transfusion of platelets or fresh frozen plasma was allowed. Informed consent was obtained from every patient no later than 24 h before treatment.

The size and number of tumor nodules were determined sonographically and by means of computerized tomography (dynamic spiral CT with *iv* application of contrast medium) prior to RFA.

Twelve consecutive patients (10 male, 2 female) with 14 tumors who met the inclusion criteria were enrolled. Mean patient age was 74 years (range 59 to 80 years). Six patients with six tumors had liver metastases from colorectal cancer, six patients with eight tumors had HCC (Table 1). All patients with HCC had underlying liver cirrhosis (Child A: $n = 4$, Child B: $n = 2$) due to chronic hepatitis C ($n = 2$) or alcohol ($n = 4$). Mean tumor size was 32 mm (range 10 to 51 mm) with two tumors up to 20 mm, six between 21 and 40 mm and six larger than 40 mm. Mean tumor diameter was 35 mm for HCC (range 24 to 45) and 30 mm for the liver metastases (range 10 to 51mm)

Table 1 Stimulation indices (SI) of each patient before, 4 and 8 wk after RFA treatment

Tumor entity	CP	Etiology of cirrhosis	SI before RFA		SI 4 wk after RFA		SI 8 wk after RFA	
			CD4	CD8	CD4	CD8	CD4	CD8
HCC 1	A	alcohol	0.012	0.017	0.086	0.125	0.15	0.146
HCC 2	A	Hep C	0.021	0.019	0.16	0.138	0.176	0.136
HCC 3	B	Hep C	0.016	0.031	0.11	0.102	0.155	0.158
HCC 4	A	alcohol	0.015	0.019	0.07	0.11	0.079	0.112
HCC 5	B	alcohol	0.026	0.021	0.069	0.118	0.099	0.139
HCC 6	A	alcohol	0.02	0.02	0.16	0.125	0.146	0.134
Mean (SD)			0.018 (± 0.005)	0.021 (± 0.005)	0.109[b] (± 0.042)	0.120[b] (± 0.012)	0.134[b] (± 0.037)	0.138[b] (± 0.015)

Tumor entity	Primary	SI before RFA		SI 4 wk after RFA		SI 8 wk after RFA	
		CD4	CD8	CD4	CD8	CD4	CD8
CRC metastasis 1	Sigma	0.025	0.023	0.123	0.158	0.129	0.155
CRC metastasis 2	Colon	0.035	0.029	0.155	0.176	0.199	0.143
CRC metastasis 3	Colon	0.01	0.017	0.069	0.11	0.126	0.125
CRC metastasis 4	Sigma	0.016	0.018	0.099	0.127	0.112	0.126
CRC metastasis 5	Rectum	0.011	0.02	0.103	0.183	0.175	0.176
CRC metastasis 6	Colon	0.02	0.021	0.14	0.147	0.149	0.137
Mean (SD)		0.019 (± 0.009)	0.021 (± 0.004)	0.115[b] (± 0.031)	0.150[b] (± 0.028)	0.148[b] (± 0.033)	0.144[b] (± 0.019)

CP: Child Pugh-Score of patients with liver cirrhosis; LC: liver cirrhosis; SI: Stimulation index, showing the ratio of IFNγ secretion of stimulated *vs* unstimulated T cells; HCC: hepatocellular carcinoma ; CRC metastasis: patients with colorectal cancer metastatic to the liver; Primary: location of the primary tumor; m.d.: missing data. [b]$P < 0.0001$ *vs* SI before RFA.

RF ablation technique

The whole procedure was performed under ultrasound guidance (Elegra Advanced®, Siemens, Erlangen, Germany) under sterile conditions. The proposed puncture site was infiltrated with a local anesthetic (2% mepivacain hydrochloride) and the perfused radio-frequency (HF) needle (Berchtold, Tuttlingen, Germany) advanced into the tumor under sonographic guidance. Midazolam (0.5-5 mg) and/or pethidin (25-100 mg) were administered iv as necessary. Patients were monitored by pulse oximetry during the entire procedure.

RFA needle applicators of 2 mm (14 G) diameter and a 15 mm active electrode with microbores were used. During HF application (40 W power output) the RFA needle was continuously perfused with isotonic saline via the bore holes. RF energy was delivered by a computer-assisted radiofrequency generator (Elektrotom 106 HF®, Berchtold, Tuttlingen, Germany) and continuous perfusion of the RFA needle was secured by a syringe pump (Pilot C, Fresenius Medical Care, Alzenau, Germany) linked to the RF generator. Perfusion was adjusted as to impedance by

means of an electronic interface between generator and perfusor, automatically increasing in response to a rise in impedance (> 400 Ohm). The RF energy was applied for 10-15 min at each needle position, leading to a coagulation zone of 30-35 mm in diameter. Tumors larger than 20 mm were targeted from different applicator positions to create overlapping coagulation zones, in order to treat the entire lesion with a safety margin > 5 mm. Larger tumors were treated with up to 3 simultaneous needle insertions arranged in a triangle (2 to 4 cm) or square (for larger tumors). After completion of the procedure patients were required to rest in bed for 4 h.

IFNγ secretion assay and lymphocyte staining

Heparinized blood (LI-Heparin 10 mL) was obtained before, 4 and 8 wk after RFA. The samples were stored at 4℃ and tests performed < 12 h after sampling. A liver biopsy of nontumorous and tumorous tissue was obtained from every patient directly before RFA and stored at -20℃.

Tissue-lysates were freshly prepared in cold phosphate buffer (50 mmol/L, pH 7.2) using a glass homogenizer as described[5]. The suspension was filtered with a filter tip (pore size 1.2 μm) to adjust the fragment size to less than 1.2 μm. The protein concentration was measured photometrically according to Bradford and adjusted to 1 mg/mL, followed by sterilization at 600 Gy.

Autologous test antigens (nontumorous liver and tumor lysate, 12.5 μg) were added to 250 μL heparinized blood and cultured in a 15 mL conical polypropylene tube for 16 h at 37℃ under 50 mL/L CO_2. A negative control without addition of antigen lysate was included, while staphylococcal enterotoxin B served as positive control antigen. Thereafter the samples were put on ice and washed with ice cold washing solution (phosphate buffer saline containing 0.5% bovine serum albumin and 2 mmol/L EDTA, pH 7.4) and the cell suspension centrifuged at 300 g for 10 min at 4℃. The cell pellet was resuspended with 80 μL cold culture medium (RPMI 1640 containing 10% human AB serum). "Catch" reagent (20 μL) containing a bivalent CD45 capture and IFNγ binding antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added, the suspension was kept for 5 min on ice and after adding 5 mL culture medium the cells were incubated in closed roller tubes for 45 min at 37℃. Thereafter 20 μL phycoerythrin (PE) labelled IFNγ detection antibodies diluted 1:5 as well as 10 μL fluoresceine-thiocyanate (FITC) labelled anti-CD 4 or anti-CD8 antibodies diluted 1:400 (both from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added to the cooled and washed cells and incubated for 10 min on ice. Five mL erythrocyte lysis buffer, containing 0.155 mol/L NH_4Cl, 10 mmol/L $KHCO_3$ and 0.1 mmol/L EDTA diluted 1:10, were added for 10 min and the cells centrifuged at 300 g for 10 min. The washed cells were resuspended in 500 μL cold washing buffer and immediately analysed in a flow cytometer (FACSCalibur, BD Biosciences, Heidelberg, Germany) after addition of 0.25 μg propidium iodide in 5 μL destilled water. FACS data were evaluated using WinMDI Version 2.8 (free version by Joseph Trotter).

T-cell conservation

PBMC containing T cells were isolated in Leukosep separation tubes by density-gradient centrifugation (PAA Laboratories GmbH, Vienna, Austria). After repeated washing with PBS (Biochrom, Berlin, Germany) containing 50 000 IU/liter heparin (Liquemin N 25 000; Roche, Grenzach-Whylen, Germany), cells were adjusted to a concentration of 10^6 cells/mL and cultured in RPMI 1640 (Biochrom) containing 10% AB serum (heat-inactivated and sterilized at 600 Gy), 5% HEPES buffer, and 1 μg/mL penicillin/streptomycin.

For further experiments 5 Mio PBMC were resuspended in 1.8 mL cold freezing medium, containing 80% AB serum and 20% DMSO (Sigma-Aldrich, Seelze, Germany), and stored at -80℃. After 24 h cells were transfered into liquid nitrogen for long term storage.

For revitalization cells were defrosted in a waterbath at 37℃ and were immediately diluted in warm culture medium (RPMI 1640 (Biochrom) containing 10% AB serum (heat-inactivated and sterilized), 5% HEPES buffer, and 1 μg/mL penicillin/streptomycin).

Cytotoxicity Assay

Cytolytic activity of T-cells was measured by an adenylate kinase (AK) release assay. 10 000 target cells were incubated with 1000 effector cells in a final volume of 200 μL fetal calf serum growth medium (FCS-GM) in round-bottom 96-well microtiter plates. After incubation for 4 h at 37℃ 100 μL of supernatant were harvested and stored at -20℃ for further analysis.

The human hepatocellular carcinoma cell line HepG2 and the human colorectal cancer cell line CaCo served as target cells for the patients suffering from HCC and colonic cancer metastases, respectively. All cell-lines were HLA matched (ABO-system) and tested before.

Maximum AK release was obtained by incubating target and effector cells with 1% v/v Triton-X-100, and baseline AK release with medium alone. Baseline release from T cells and tumor cells was < 10% of maximal release in all experiments and subtracted from each value.

The activity of AK was determined by detection of autoluminescence using a luciferase assay (ToxiLight Kit, Cambrex Corporation, NJ, USA). Twenty μL of supernatant were incubated with AK-detection reagent (Cambrex Corporation, NJ, USA) for 5 min at room temperature. The bioluminescence was measured by a luminometer (BD Monolight 3096 Microplate Luminometer, BD Biosciences, Heidelberg, Germany) and expressed as relative luminescence units (RLU).

Statistical analysis

The stimulation index of $CD4^+$ and $CD8^+$ T cells (SI) was determined by the ratio of $IFN\gamma^+$ *vs* $IFN\gamma^-$ $CD4^+$ and $CD8^+$ T cells, respectively, and calculated with Excel® 2000 software (Microsoft, Seattle, USA). Results were analyzed statistically by the SPSS® software package (Version 11.01 d, SPSS GmbH Software, Munich, Germany). SI values were divided into 2 groups according to the histological origin of the tumors. SI values were calculated numerically (Table 1) and are presented as Box-Whisker plots (Figure 1). The

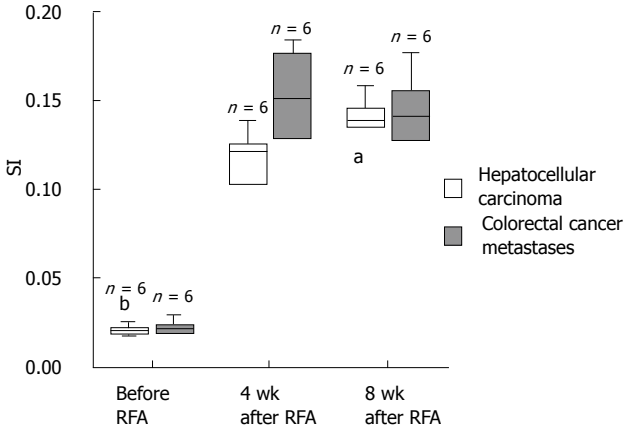


Figure 1 Stimulation indices of $CD8^+$ T cells. The Box-Whisker-plots show the ratio of IFNγ secretion of stimulated *vs* unstimulated $CD8^+$ T cells as determined by a cytokine secretion and capture assay before, 4 and 8 wk after RFA. White boxes represent the patients with HCC and grey boxes the patients with colorectal cancer metastases. Outlayers are marked with "a", extremes with "b". For significances refer to table 1.

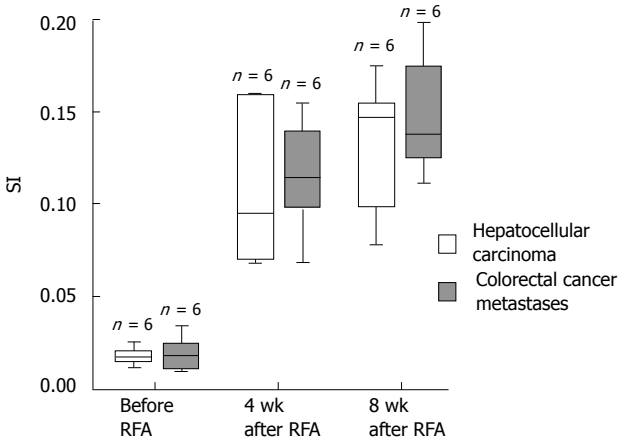


Figure 2 Stimulation indices of $CD4^+$ T cells. The Box-Whisker-plots show the ratio of IFNγ secretion of stimulated *vs* unstimulated $CD4^+$ T cells as determined by a cytokine secretion and capture assay before, 4 and 8 wk after RFA. White boxes represent the patients with HCC and grey boxes the patients with colorectal cancer metastases. Outlayers are marked with o, extremes with *. For significances refer to table 1.

significance of the enhanced SI after RFA treatment was tested with the Wilcoxon test for dependent samples. A *P*-value less than 0.05 was considered significant.

RESULTS

A total of 26 needle insertions were performed for 12 patients, i.e., 2.2 sessions per patient (range 1 to 3 sessions). Complete remission was achieved in all 12 patients. 2 patients developed a local recurrence within 4 months. One patient could be successfully retreated. The other patient with liver metastases from colorectal carcinoma could not be retreated successfully. Distant recurrences did not occur within the mean follow up-period of 6 months.

The cytokine secretion and capture assay showed that autologous nontumorous control liver tissue did not stimulate IFNγ secretion of circulating T cells before or after RFA. Before RFA: $SI_{[CD4+]} = 0.018$ (± 0.007) and $SI_{[CD8+]} = 0.019$ (± 0.004); 4 wk after RFA $SI_{[CD4+]} = 0.017$ (± 0.005) and $SI_{[CD8+]} = 0.019$ (± 0.008); 8 wk after RFA: $SI_{[CD4+]} = 0.018$ (± 0.006) and $SI_{[CD8+]} = 0.016$ (± 0.007). When exposed to autologous tumor tissue all patients displayed a mean $SI_{[HCC\ CD4+]}$ of 0.018 (± 0.005) and $SI_{[CRC\ CD4+]}$ of 0.019 (± 0.009) and $SI_{[HCC\ CD8+]}$ of 0.021 (± 0.005) and $SI_{[CRC\ CD8+]}$ of 0.021 (± 0.004) before RFA (Table 1).

Four wk after RFA all patients showed a strong increase of $IFN\gamma^+$ and $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells directed against autologous tumor tissue ($SI_{[HCC\ CD4+]} = 0.109$ (± 0.042); $SI_{[HCC\ CD8+]} = 0.120$ (± 0.012); $SI_{[CRC\ CD4+]} = 0.115$ (± 0.031); $SI_{[CRC\ CD8+]} = 0.150$ (± 0.028)). This increase was highly significant both for the patients with HCC and with colorectal cancer metastases ($P < 0.0001$) and was maintained until 8 wk after RFA (Table 1, Figures 1 and 2).

Four wk after RFA PBMC of both groups, HCC and metastasis patients, presented a highly elevated cytotoxic activity with a mean release of adenylate cyclase (AK, as represented by RLU) of 580.24 (± 190.81) and 677.15 (± 138.38) for HCC and colorectal cancer metastasis, respectively. This was maintained 8 wk after RFA with RLU values of 784.96 (± 181.73) and 954.59 (± 275.42). All data were highly significant compared to the baseline before RFA, with 4.42 RLU (± 0.68) for HCC and 2.62 RLU (± 0.37) for colorectal cancer metastasis ($P < 0.001$). The spontaneous release of AK was always < 2% of the maximum release (Figure 3 and Table 2).

DISCUSSION

Thermal treatments such as RFA or LiTT are increasingly used in the therapy of unresectable liver tumors. Survival of treated patients is encouraging. Thus for patients with liver metastases of colorectal cancer, 2 and 5 year survival rates after LiTT of 74% and 30%, respectively,[2, 3], and a 2 year survival of 67% after RFA[6] were reported. These data are comparable with results of surgical resection that can lead to 2 and 5 year survival rates of 64% and 28%, respectively, and to disease free survival rates of 35 and 15% for patients with liver metastases of colorectal cancer. Similar results have been obtained for patients with HCC[7, 8]. Here again, minimal invasive treatments seem to offer the same survival benefit as open surgery[9,10]. It appears improbable that the achieved results can merely be explained by physical effects like thermal tumor ablation or by a favourable patient selection in case of the nonsurgical group, since patients treated with RFA (or LITT) are often not eligible for hepatic resection due to poor liver function or advanced tumor disease.

Immunotherapy through T cell vaccination or vaccination with dendritic cells is regarded as a promising strategy for the treatment of various tumor entities such as malignant melanomas[11-16], and high expectations have been placed on cytokine-modulated immunotherapies for hepatic tumors[17,18]. It is well known that RFA can induce an unspecific immune stimulation[4]. Thus thermal coagulation causes an inflammatory reaction with lymphoplasmacellular infiltration that can be visualized as a hypervascular rim in contrast CT and contrast-enhanced ultrasound[19]. This

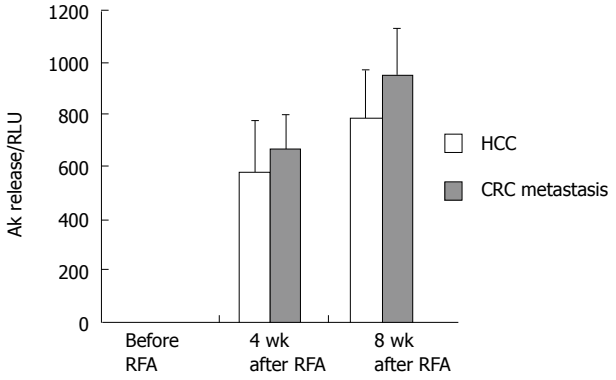


Figure 3 Adenylate kinase (AK) release of cultured tumor cells. Release of adenylate kinase (AK) from the human hepatoma cell line HepG2 and the hman colorectal carcinoma cell line CaCo co-incubated with PBMC isolated before, 4 and 8 wk after RFA. The release of AK correlates with the cytotoxic and NK T cell mediated damage to the cultured cells. HepG2 cells were used for PBMC from the patients with HCC and CaCo cells for the patients with metastasis of colorectal carcinoma.

hypervascular rim can be so intense as to impede proper assessment of treatment success.

We could recently demonstrate a specific T cell response towards the VX2-tumor implanted in rabbit livers after RFA was performed[5]. The VX2 hepatoma is a papilloma virus-induced tumor[20] which contains viral genomes[21] that are immunogeneic themselves. However, the vigorous T cell reaction was shown not to be directed against viral, but the non-viral tumor antigens. Thus the significantly prolonged survival of RFA-treated animals observed in preliminary studies[5,22,23] supports the hypothesis that tumor immunity induced by in situ vaccination via thermal necrosis may play an important role in the clinical effect of RFA.

It remained to be proven if RFA induces a tumor specific T cell reaction also in humans with hepatic tumors. In the present preliminary study we could clearly demonstrate that patients with HCC or colorectal cancer metastases experienced a 5-6 fold rise of peripheral tumor-specific IFNγ^+, CD4$^+$ T helper cells and CD8$^+$ T killer or cytotoxic T cells[24] 4 wk after RFA. Furthermore, this effect was maintained until 8 wk after RFA. The cytotoxic activity of the T cells derived from PBMC showed a dramatical, up to 300 fold increase compared to baseline already 4 and 8 wk after RFA as well.

Two patients with local recurrence (patient HCC4 and CRC 4 in Table 1 and 2) displayed a lower cytotoxic activity against the cancer cells (96.8 and 157.7 RLU 8 months after RFA), as compared to 2 patients without recurrence (patient HCC 2 and CRC 5 with 739.4 and 1004.6 RLU 12 mo after RFA). Due to the low number of cases this observation is not significant and has to be confirmed in a larger group of patients.

Thus it appears that the coagulation of tumor tissue through RFA leads to the enhanced release, exposure and/or denaturation of tumor antigens. Thermally altered tumor antigens are more likely to be phagocytosed by professional antigen presenting cells like dendritic cells[25]. In conjunction with the generation of thermally altered tumor antigens the unspecific inflammatory stimulus induced by RFA might help to overcome immune-tolerance or anergy towards the transplanted tumor, a common phenomenon observed in solid tumors[26,27]. The pro-inflammatory effects of necrotic cells are well documented and appear to be caused by the release of endogenous adjuvants, such as the nuclear protein high mobility group B1 (HMGB1) and heat shock proteins such as hsp70 or gp96[28-30]. RFA thus appears to create an in situ environment resembling T cell vaccination ex vivo.

Our study does not permit conclusions as to whether the stimulation of tumor-specific T cells was solely due to the creation of thermal necrosis, to the instillation of hot saline solution or to the application of a high-frequency current. The relevance and the impact of RFA-induced generation of tumor-specific cytotoxic and NK T cells for potential future multimodal antitumor therapies has to be investigated in prospective randomized multi center trials.

Table 2 Relative luminescence units (RLU) of cytosolic adenylate kinase released from the hepatoma cell line HepG2 for HCC patients and the colorectal carcinoma cell line CaCo for patients with colorectal carcinoma metastatic to the liver

Tumor entity	CP	Etiology of cirrhosis	AK release before RFA	AK release 4 wk after RFA	AK release 8 wk after RFA
HCC 1	A	alcohol	4.8	398.46	535.87
HCC 2	A	Hep C	3.26	421.66	645.9
HCC 3	B	Hep C	4.88	578.63	845.99
HCC 4	A	alcohol	3.95	865.66	1025.21
HCC 5	B	alcohol	5	465.7	732.13
HCC 6	A	alcohol	4.66	751.33	924.66
Mean			4.42	580.24[b]	784.96[b]
(SD)			0.68	190.81	181.73

Tumor entity	Primary	AK release before RFA	AK release 4 wk after RFA	AK release 8 wk after RFA
CRC metastasis 1	Sigma	2.13	568.32	816.55
CRC metastasis 2	Colon	3	947.47	1298.25
CRC metastasis 3	Colon	2.79	627.55	945.33
CRC metastasis 4	Sigma	2.56	699.45	845.54
CRC metastasis 5	Rectum	2.98	598.65	932.17
CRC metastasis 6	Colon	2.27	621.46	889.66
Mean		2.62	677.15[b]	954.58[b]
(SD)		0.37	139.38	175.42

Cells were co-incubated with PBMC isolated from each patient taken before, 4 and 8 wk after RFA. CP: Child Pugh-Score of patients with liver cirrhosis; LC: liver cirrhosis; HCC: hepatocellular carcinoma; CRC metastasis: patients with colorectal cancer metastastatic to the liver; Primary: location of the primary tumor; m.d.: missing data; [b]$P < 0.0001$ *vs* bioluminescence before RFA.

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VIRAL HEPATITIS

Mutations in carboxy-terminal part of E2 including PKR/eIF2α phosphorylation homology domain and interferon sensitivity determining region of nonstructural 5A of hepatitis C virus 1b: Their correlation with response to interferon monotherapy and viral load

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Abstract

AIM: To study the amino acid substitutions in the carboxy (C)-terminal part of E2 protein and in the interferon (IFN) sensitivity determining region (ISDR) and their correlation with response to IFN and viral load in 85 hepatitis C virus (HCV)-1b-infected patients treated with IFN.

METHODS: The C-terminal part of E2 (codons 617-711) including PKR/eIF2α phosphorylation homology domain (PePHD) and ISDR was sequenced in 85 HCV-1b-infected patients treated by IFN monotherapy.

RESULTS: The amino acid substitutions in PePHD detected only in 4 of 85 patients were not correlated either with response to IFN or with viral load. The presence of substitutions in a N-terminal variable region (codons 617-641) in the C-terminal part of E2 was significantly correlated with both small viral load (33.9% *vs* 13.8%, $P = 0.0394$) and sustained response to IFN (25.0% *vs* 6.9%, $P = 0.0429$). Four or more substitutions in ISDR were significantly correlated with both small viral load (78.6% *vs* 16.2%, $P < 0.0001$) and sustained response to IFN (85.7% *vs* 2.9%, $P < 0.0001$). In multivariate analysis, ISDR in nonstructural (NS) 5A ($OR = 0.39$, $P < 0.0001$) and N-terminal variable region ($OR = 0.51$, $P = 0.039$) was selected as the independent predictors for small viral load, and ISDR ($OR = 39.0$, $P < 0.0001$) was selected as the only independent predictor for sustained response.

CONCLUSION: The N-terminal variable region in the C-terminal part of E2 correlates with both response to IFN monotherapy and viral load and is one of the factors independently associated with a small viral load.

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Key words: E2; Genotype; HCV; Interferon; ISDR; NS5A; PePHD; PKR; SVR

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INTRODUCTION

Hepatitis C virus (HCV) is one of the most frequent and important causes of chronic viral hepatitis and approximately 170 million people are infected with the virus worldwide[1]. HCV usually causes chronic infection, which can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma[2].

Interferon (IFN) monotherapy and combination therapy of IFN and ribavirin have been shown to be effective for eradicating chronic HCV infection[3,4]. Genotype 1b is resistant against antiviral therapy[5], and viral load is known as a predictor for response to IFN mono therapy[6]. While the combination therapy has significantly improved the effectiveness for patients with large viral load of genotype 1b, viral load still determines

the outcome of treatment[7].

RNA-activated protein kinase (PKR) is a mediator of IFN-induced antiviral resistance. PKR-binding domain (PKR-bD) comprising the interferon sensitivity determining region (ISDR) within the nonstructural (NS) 5A protein is known to be potentially important for antiviral therapy outcome. However, reported results concerning the correlation of the substitutions within PKR-bD/ISDR to treatment outcome are conflicting[8-12].

A PKR/eIF2α phosphorylation homology domain (PePHD, codons 659-670) within the carboxy (C)-terminal part of E2 protein has been found to interact with PKR and inhibit PKR in vitro, suggesting a possible mechanism of HCV to evade the antiviral effects of IFN[13]. Several studies have concluded that a correlation of substitutions in PePHD does not exist[14-17], while only 3 others have confirmed it[18-20]. PePHD-deleted HCV-1 mutants remain capable of binding to PKR to some extent, while C-terminal-truncated E2 protein loses activity of inhibiting PKR. Thus regions other than PePHD within C-terminal part of E2 protein may interact with PKR. It is possible that the mutations in regions other than PePHD within C-terminal part of E2 protein may correlate with response to IFN. Sarrazin *et al*[21] reported that substitutions in C-terminal part of E2 are linked with response to antiviral therapy.

In the present study, we investigated the amino acid substitutions in the C-terminal part of E2 protein and in ISDR and their correlation with response to IFN and viral load in 85 HCV-1b infected patients treated with IFN.

MATERIALS AND METHODS

Patients

Eighty-five patients infected with HCV genotype 1b, were retrospectively analyzed (Table 1). The patients received various IFN preparations in various schedules of administration. Thirty-six patients received lymphoblastoid IFNα, 14 IFNα 2a, 18 natural IFNβ, 3 IFNα 2b and 14 both lymphoblastoid IFNα and natural IFNβ. Total dose of IFN was 200-400MU in 32, 400-600MU in 40, and 600MU or more in 13 patients. All patients were positive for both serum anti-HCV antibody (second generation enzyme immunoassay, Abbott laboratories, North Chicago, IL) and serum HCV RNA (Amplicor HCV test, Nippon Roche, Tokyo). HCV genotype was determined as previously described[22] and classified according to Simmonds' the classification of simmonds *et al*[23]. Viral load was measured by branched DNA assay (Quantiplex HCV RNA assay, version 2, Chiron Corp., Emeryville, CA). Histological evaluations were done according to the grading and staging suggested by Desmet *et al*[24].

Patients who obtained normalization of alanine aminotransferase (ALT) levels and HCV RNA clearance for more than 6 mo after IFN therapy were considered as sustained virological responders (SVR). The other patients were grouped as nonresponders.

This study was approved by the local committee on medical ethics and adhered to the guidelines of the 1975 Declarations of Helsinki.

Table 1 Characteristics of patients with different response to IFN therapy

	Sustained responders	Non-responders	*P*
Patients (*n*)	16	69	
Age (yr)	51.2 ± 10.7	52.4 ± 11.1	NS
Male/Female	14/2	47/22	NS
History of blood transfusion (+/-)	5/11	21/48	NS
ALT (IU/l)	110.4 ± 88.0	95.6 ± 61.0	NS
Viral load (Meq/mL)	0.9 ± 0.9	6.9 ± 7.8	0.0027
Histology			
Grade	1.9 ± 0.7	2.3 ± 0.8	NS
Stage	1.9 ± 0.7	2.5 ± 0.9	0.0154
Number of amino acid Substitutions in			
ISDR	5.0 ± 1.2	0.7 ± 1.1	< 0.0001
C-terminal part of E2	3.4 ± 1.7	2.5 ± 1.2	0.0149
N-terminal variable region	1.6 ± 1.1	0.9 ± 0.9	0.0148
Substitution of aa 625 (+/-)	6/10	8/61	0.0213

Sequencing of the C-terminal part of E2 protein comprising PePHD

HCV RNA was extracted from 100 μL of patients' sera before IFN therapy by Sepa Gene RV-R™ (Sanko Junyaku Co. Inc., Tokyo). cDNA was prepared from extracted RNA by reverse transcription using the random primer (Takara, Ohtsu, Japan). Nested polymerase chain reaction (PCR) was done for amplifying the C-terminal part of the E2 protein comprising PePHD (nt 2178-2462). Four primers designed from the nucleotide sequences of HCV-J were used for the nested PCR. The 1st round of PCR was performed using the external primers (sense primer; nt 2127-2143; 5' -GGGCCCTGGTTGACACC-3', and anti-sense primer; nt 2508-2524; 5'-GCGGACGCGCGCGTCTG-3') and the 2nd round of PCR was performed using the internal primers (sense primer; nt 2157-2174; 5' -GACTACCCATACAGGCTC-3', and anti-sense primer; nt 2496-2515; 5'-TTCCTTCTTCTGGCGGA-3'). All rounds of PCR were done with Taq polymerase (Takara, Ohtsu, Japan) by the following protocol: 35 cycles of denaturation for 1 min at 95℃, annealing for 1 min at 55℃, and extension for 1 min at 72℃, and a final extension for 7 min at 72℃.

Sequencing was performed by the PCR direct sequencing method. Amplified DNA was isolated by agarose gel electrophoresis and purified by EASYTRAP™ Ver.2 (Takara, Ohtsu, Japan). The nucleotide sequence was determined by the dideoxy method using a dye terminator cycle sequencing kit (Perkin-Elmer Japan, Tokyo, Japan).

Sequencing of ISDR in NS5A

cDNA of ISDR in NS5A was prepared by reverse transcription using 5ASRAW primer (nt 7177-7197; 5'-GT GCCCATATGGGCAACGCTG-3') with RNA extracted from 50 μL of serum and then amplified by nested PCR with a DNA thermal cycler (Lobocycler 40; Stratagene, La Jolla, CA). The 1st round of PCR was performed using the external primers (sense primer 5SPDV; nt 6825-6844; 5'-C

CGGATGTGGCAGTGCTCAC-3', and anti-sense primer 5ASRAW. The second round of PCR was performed using the internal primers (sense primer 5STDV; nt 6853-6874; 5 '-TCACCGACCCCTCTCATATTAC-3', and biotin-labeled anti-sense primer 5IASPKR; nt 7141-7161; 5'-GTTTTCGC AGGATCTCCGCCG-3'). Twenty-nine cycles of PCR were done as follows: denaturation for 1 min at 94℃, annealing for 1.5 min at 56℃, and extension for 1.5 min at 72℃. The PCR products labeled with biotin were recovered with streptavidin-coated magnetic beads (Dynal, Oslo, Norway). The sequencing reaction was performed using a thermo sequenase fluorescent-labeled primer cycle sequencing kit (Amersham, Buckinghamshire, England) with Cy5-labeled 5ISKRR fluorescent primer (nt6887-6906; 5'-CA AGCGTAGGCTGGCCAGGG-3') under the conditions recommended by the kit supplier. The reaction mixture was applied to the ALFII DNA sequencer (Amersham Pharmacia Biotech, Tokyo, Japan). Identification of the substituted amino acid was performed using a KINOP alignment software system (Otsuka-Fujitsu Collaborative Development, Tokyo, Japan).

Phylogenetic tree construction

A phylogenetic tree was constructed to determine the genetic relation using the Neighbour-joining method[25].

Statistical analysis

Data were analyzed by Student's *t* test, chi-square test, and linear regression test for each applicable analysis. Multivariate analysis was done by discriminant analysis by stepwise forward selection method. $P < 0.05$ was considered statistically significant.

RESULTS

Response to IFN therapy

Sixteen of 85 patients had sustained virological response (Table 1). Viral load was significantly smaller in sustained virological responders than in non-responders ($P = 0.0027$). Stage of liver histology was significantly lower in sustained virological responders than in non-responders ($P = 0.0154$).

Sequences of C-terminal part of E2 protein

All of the obtained sequences were aligned. The most popular amino acid at each position was determined as the consensus sequence. Since there were two amino acids (isoleucine and valine) equally popular at codon 705, we considered both of them as the consensus sequences. The number of amino acid substitutions in the C-terminal part of E2 protein (codons 617-711) was 0 in 2, 1 in 13, 2 in 30, 3 in 22, 4 in 10, 5 in 3 and 6 in 5 patients respectively (Figures 1A and B).

Amino acid substitutions in PePHD were seen only in 4 out of 85 patients and did not correlate either with viral load or with response to IFN (Figures 1A and B).

The number of substitutions in the C-terminal part of E2 protein was significantly larger in sustained virological responders than in non-responders ($P = 0.0149$, Table 1). The patients with 4 or more substitutions in the C-terminal part of E2 protein had sustained virological response to IFN therapy [38.9% (7/18)] more frequently than those with less than 4 substitutions [13.4% (9/67)]($P = 0.0358$).

In the molecular-evolutional analysis with phylogenetic tree, we could not find any specific clustering either in sustained virological responders or in non-responders (Data not shown).

Table 2 Characteristics of patients with small or large viral load

	Patients with small viral load	Patients with large viral load	*P*
Patients (*n*)	24	61	
Age (yr)	52.5 ± 11.0	52.0 ± 11.1	NS
Male/female	19/5	42/19	NS
History of blood transfusion (+/-)	8/16	18/43	NS
ALT (IU/I)	89.3 ± 64.1	102.0 ± 67.6	NS
Viral load (Meq/mL)	0.5 ± 0.1	7.8 ± 7.9	0.0001
Histology			
Grade	2.2 ± 0.8	2.3 ± 0.8	NS
Stage	2.3 ± 0.8	2.4 ± 0.9	NS
Number of amino acid Substitutions in			
ISDR	3.3 ± 2.5	0.8 ± 1.2	< 0.0001
C-terminal part of E2	3.1 ± 1.7	2.5 ± 1.2	NS
N-terminal variable region	1.5 ± 1.0	0.9 ± 0.9	0.0090
Substitution of aa 625 (+/-)	7/17	7/54	NS

N-terminal region in the C-terminal part of E2 protein

In the C-terminal part of E2 protein, there were four variable regions where amino acid sequences were variable (Figures 1A and B). The regions correlating with response to IFN and viral load were searched for. The number of substitutions in N-terminal variable region designated as variable region 1 was significantly larger in sustained virological responders than in non-responders ($P = 0.0148$, Table 1). The patients with substitutions in N-terminal variable region had sustained virological response to IFN therapy [25.0% (14/56)] more frequently than those without substitutions [6.9% (2/29), $P = 0.0429$]. The number of substitutions in N-terminal variable region was significantly larger in the patients with small viral load (HCV-RNA < 1Meq/mL) than in those with large viral load ($P = 0.0107$, Table 2). The patients with substitutions in N-terminal variable region had small viral load [33.9% (19/56)] more frequently than those without substitutions [13.8% (4/29), $P = 0.0394$]. Amino acid substitutions in other parts of C-terminal of E2 protein were not correlated with either viral load or sustained response to IFN therapy (Data not shown).

Specific amino acid substitutions correlated with viral load or with response to IFN therapy were searched for. Amino acid substitutions of threonine to serine at codon 625 were found to be significantly correlated with sustained virological response to IFN therapy ($P = 0.0213$) (Table 1).

Changes in sequences of C-termianl part of E2 after IFN therapy

The sequences of C-terminal part of E2 after IFN therapy

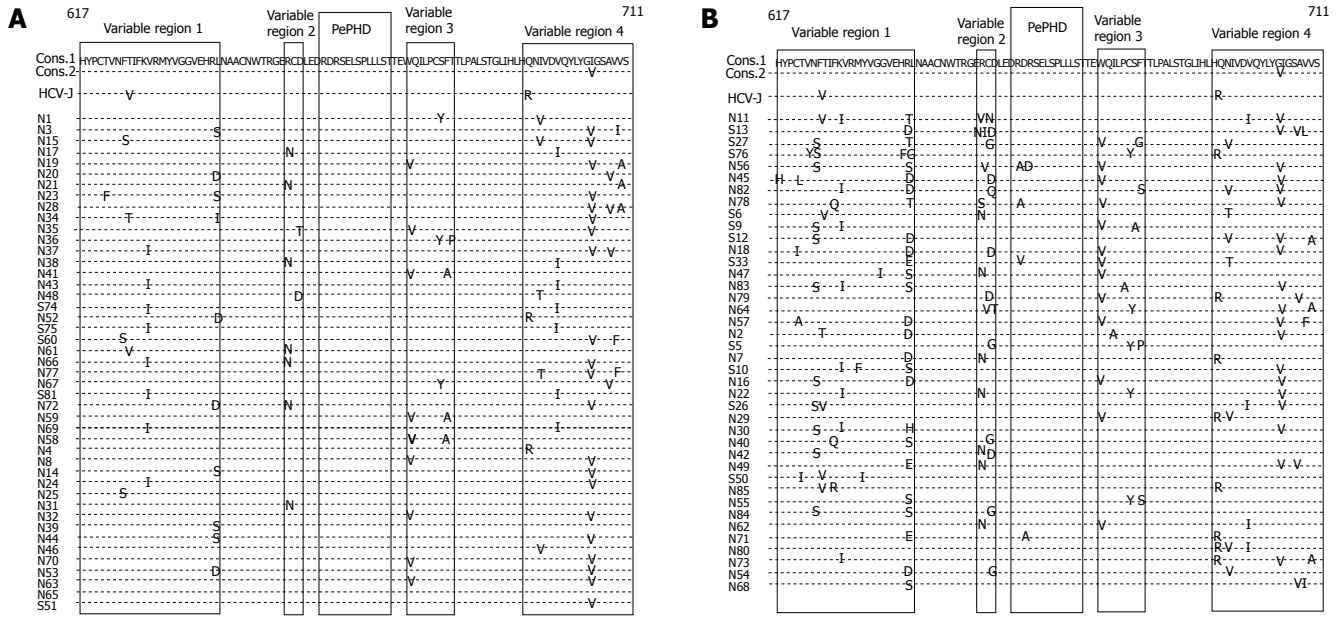


Figure 1 Amino acid sequences of C-terminal part of E2 protein with 0-2 substitutions (**A**) and 3-6 substitutions (**B**) in 45 and 40 HCV 1b-infected patients treated by IFN therapy respectively.

were determined in 16 non-responders (Figure 2). In 8 patients, the sequences did not change before and after IFN therapy, while in the other 8 patients, the sequences changed. The number of substitutions in C-terminal part of E2 decreased in 3 patients (from 5 to 2 in N45, from 3 to 2 in N7, and from 2 to 1 in N3), increased in 4 patients (from 2 to 3 in N28 and N36, and from 1 to 3 in N31 and N46), and did not changed in N38.

Sequences of ISDR

The sequences of ISDR were determined in 82 patients (Figure 3). The number of substitutions in ISDR was significantly larger in sustained virological responders than in non-responders ($P < 0.0001$, Table 1). The patients with 4 or more substitutions in ISDR had sustained virological response to IFN therapy [85.7% (12/14)] more frequently than those with less than 4 substitutions [2.9% (2/68), $P < 0.0001$]. The number of substitutions in ISDR was significantly larger in the patients with small viral load than in those with large viral load ($P < 0.0001$, Table 2). The patients with 4 or more substitutions in ISDR had small viral load [78.6% (11/14)] more frequently than those with less than 4 [16.2% (11/68), $P < 0.0001$].

Correlation between substitutions in C-terminal part of E2 and ISDR

The substitutions in C-terminal part of E2 and ISDR were significantly correlated with each other ($P = 0.026$, $r = 0.246$) (Figure 4). The number of substitutions in N-terminal variable region did not correlate with that in ISDR.

Multivariate analysis for factors independently associated with response to IFN therapy and viral load

In multivariate analysis, the number of substitutions in ISDR was selected as the only independent factor

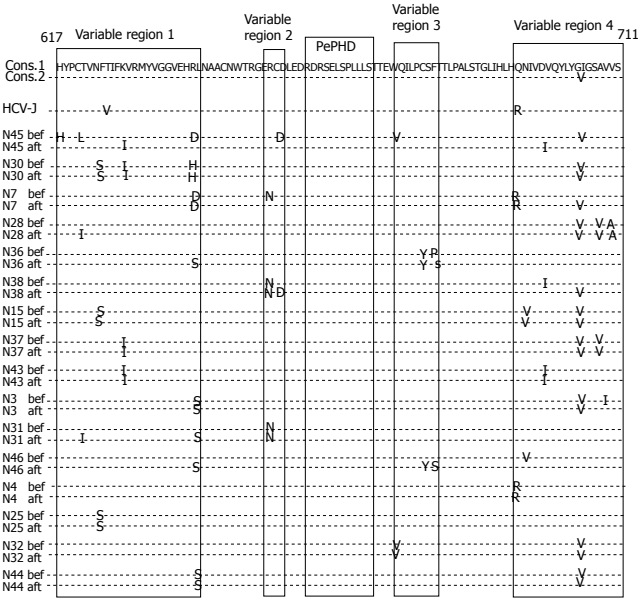


Figure 2 Amino acid sequences of C-terminal part of E2 before and after IFN therapy in 16 non-responders.

associated with sustained virological response to IFN therapy ($OR = 39.0$, $P < 0.0001$). The substitutions in ISDR ($OR = 0.39$, $P < 0.0001$) and N-terminal variable region ($OR = 0.51$, $P = 0.039$) were selected as the independent factors correlated with small viral load.

DISCUSSION

In the present study, the substitutions in C-terminal part of E2 were found to be significantly correlated with response to IFN therapy. Especially, the substitutions in N-terminal variable region were significantly correlated with response to IFN therapy and one of the factors independently

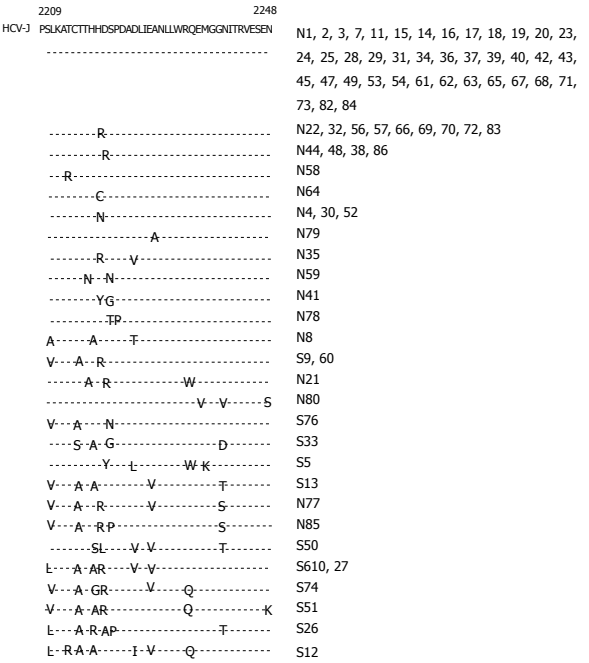


Figure 3 Amino acid sequences of ISDR in 82 HCV-infected patients.

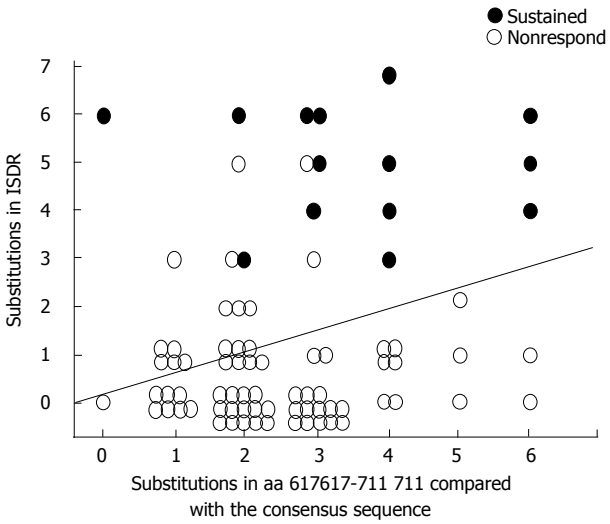


Figure 4 Correlation between the number of amino acid substitutions in ISDR and C-terminal part of E2 protein.

associated with small viral load. So far, only Sarrazin *et al*[21] have suggested the correlation of the substitutions in C-terminal part of E2 with response to antiviral therapy. They reported that normalized Shannon entropy as well as mean Hamming distances in C-terminal part of E2 are significantly lower in sustained responders than in non-responders by calculation of genetic complexity and diversity of amino acid sequences, although no significant correlation of specific mutations or number of mutations within C-terminal part of E2 with response to IFN therapy has been found[21]. Further studies are needed to assess the correlation of sequences of C-terminal part of E2 with response to the combination therapy of IFN and ribavirin as well as viral load.

Tayler *et al*[13] described that PePHD-deleted HCV-1 mutants remain capable of binding to PKR to some extent, while C-terminal-truncated E2 protein loses activity of inhibiting PKR. This finding indicates that regions other than PePHD within C-terminal part of E2 interact with PKR and play an important role in suppressing PKR, a mediator of IFN-induced antiviral resistance. The correlation of C-terminal part of E2 with response to IFN therapy and small viral load may suggest that the substitutions in the region abrogate the activity of suppressing PKR, resulting in susceptibility to IFN therapy and small viral load.

When the sequences of C-terminal part of E2 were compared before and after IFN therapy, the decrease in substitutions was observed in only 3 of 16 patients, indicating that resistant HCV becomes predominant after IFN therapy. The factors other than the number of substitutions of C-terminal part of E2 also play a role in resistance to IFN therapy.

The present study showed that PePHD was highly conserved and that there were no significant correlations of the substitutions of PePHD with the response to IFN therapy or with viral load. It was reported that PePHD has similar sequences with PKR and eIF2α in E2 region of HCV genotype 1a[13]. Other studies also showed that PePHD is highly conserved and that there are no significant correlations between the substitutions in PePHD and the response to IFN therapy[14-17]. Only three reports on genotypes 1, 2, and 3 have reported a correlation between substitutions of PePHD and the effect of IFN therapy[18-20]. Recently Bagaglio *et al*[26] reported that substitutions in PePHD correlate with hepatocellular carcinoma. Further studies on the correlation of substitutions of PePHD with clinical aspects of chronic HCV infection are needed.

In the present study, the substitution of threonine to serine at codon 625 significantly correlated with sustained response. No reports are available on the correlation of a specific amino acid substitution with response to IFN therapy. Both threonine and serine have side chains containing hydroxylic groups which are similar to each other. Thus this substitution makes only a slight difference in the structure of E2 protein. Further studies are necessary to elucidate the correlation of the substitution with response to IFN therapy.

The correlation of ISDR with viral load and response to IFN therapy was confirmed in the present study. Gale *et al*[27] reported that NS5A represses PKR through direct interaction and inhibits IFN-induced antiviral response. Multiple ISDR mutations probably abrogate this action of NS5A on inhibiting PKR[28]. This is probably the reason why patients with mutant ISDR are likely to obtain sustained virological response and have small viral load. Inhibition of antiviral activity of IFN by NS5A has been shown in mammalian cells using encephalomyocarditis virus and vesicular stomatitis virus[29]. However inconsistent association between type of ISDR and IFN sensitivity *in vitro* has been also reported[30].

The present study demonstrated the correlation between the number of substitutions in the C-terminal part of E2 and ISDR. Both regions bind to PKR and inhibit its activity. It may be the reason why the mutations

of the two regions occur simultaneously. They are probably adoptive mutations to the circumstances of individual hosts. The mutations may bring out some advantages for HCV to survive, while they may also make HCV susceptible to IFN or viral load smaller. Maekawa *et al*[31] reported that introduction of NS5A mutations enables HCV replicon derived from HC-J4 to replicate efficiently in Huh-7 cells[31]. The possible advantages of the mutations in the two regions require further elucidation. The mutations in ISDR also have been reported to correlate with development of hepatocellular carcinoma[32]. The role of the C-terminal part of E2 in the development of hepatocellular carcinoma is needed to be future studied.

In conclusion, N-terminal variable region in the C-terminal part of E2 correlates with both response to IFN therapy and viral load, and it is one of the factors independently associated with small viral load.

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BASIC RESEARCH

Anti-inflammatory effects of bifidobacteria by inhibition of LPS-induced NF-κB activation

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Abstract

AIM: Different strains of bifidobacteria were analysed for their effects on HT-29 intestinal epithelial cells (IECs) in *in vitro* models both of the non-inflamed and inflamed intestinal epithelium.

METHODS: A reporter gene system in HT-29 cells was used to measure levels of NF-κB activation after challenge with bifidobacteria or after bacterial pre-treatment following LPS challenge. IL-8 protein and pro-inflammatory gene expression was investigated using normal HT-29 cells.

RESULTS: None of the bifidobacteria tested induced activation of nuclear factor κB (NF-κB) indicating that bifidobacteria themselves do not induce inflammatory events in IECs. However, six out of eight bifidobacteria tested inhibited lipopolysaccharide- (LPS-) induced NF-κB activation in a dose- and strain-dependent manner. In contrast, NF-κB activation in response to challenge with tumor necrosis factor-α (TNF-α) was affected by none of the tested bifidobacteria, indicating that the inhibitory effect of bifidobacteria is specific for LPS-induced inflammation in IECs. As shown with two of the six inhibition-positive bifidobacteria, LPS-induced inhibition of NF-κB activation was accompanied by a dose-dependent decrease of interleukin 8 (IL-8) secretion and by lower mRNA levels for IL-8, TNF-α, cyclooxygenase 2 (Cox-2), and intercellular adhesion molecule 1 (ICAM-1).

CONCLUSION: Some strains of bifidobacteria are effective in inhibiting LPS-induced inflammation and thus might be appropriate candidates for probiotic intervention in chronic intestinal inflammation.

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INTRODUCTION

Inflammatory bowel diseases such as Crohn's disease (CD) and ulcerative colitis (UC) are multifactorial disorders characterized by chronic inflammation of the intestinal epithelium. In addition to the genetic predisposition[1], the intestinal microflora seems to be involved in triggering inflammation, as observed in several animal models, where colitis was not induced in germ-free animals[2-4]. It has been suggested, that breakdown of tolerance towards the intestinal bacterial flora and dysfunction of regulatory T-cells are implicated in the development of chronic intestinal inflammation[5-8].

One of the central transcription factors mediating inflammatory responses is nuclear factor κB. Upon activation, through a wide range of stimuli such as TNF-α, IL-1β, or LPS, NF-κB translocates to the nucleus[9,10], where it regulates the transcription of a series of genes involved in acute responses to injury and in chronic intestinal inflammation including the genes for IL-1β, TNF-α, IL-6, IL-8, IL-12, Cox-2, ICAM-1, vascular endothelial growth factor-1, T-cell receptor-α, and major histocompatibility complex class II molecules[11,12]. LPS activates NF-κB through toll-like receptor 4 (TLR4)[13]. The events preceding the actual binding of LPS to TLR4 involve LPS-binding protein (LBP) and the intermediate receptor CD14[14,15]. LBP conveys LPS to CD14, which then promotes binding of LPS to the LPS receptor complex composed of TLR4 and MD-2, a co-receptor essential for LPS signalling via TLR4[16]. On monocytes and macrophages CD14 was shown to be membrane-bound by a glycosylphophoinositol anchor[17]. Stimulation of endothelial and epithelial cells with LPS requires the second form of CD14, i.e. soluble CD14 (sCD14)[18].

There is increasing evidence that in chronic intestinal inflammation expression of TLR4 and CD14 on IECs is abnormal. Under normal conditions, TLR4 and CD14

are expressed in IECs at very low levels *in vivo*[19,20] and are confined to undifferentiated cells of the crypts, which are not exposed to the overwhelming quantity of luminal antigens[21]. By contrast, expression of TLR4 and CD14 is significantly increased in IECs of both, animal models of colitis[22,23] and IBD patients[19]. A possible explanation for this increased expression could come from genetic studies. Higher frequencies of the Asp299Gly allele in the extracellular domain of TLR4 and the T allele and TT genotype at position -159 in the gene for CD14 have been positively associated with CD and UC[24-27]. Abnormal LPS signalling through increased levels of TLR4 and CD14 in IECs could contribute to the sustained mucosal inflammation in IBD.

Bifidobacteria are Gram-positive, anaerobic micro-organisms that inhabit mainly the colon of healthy infants and adults. In breast-fed infants, soon after birth, up to 90% of all bacteria in faecal samples detected by fluorescence *in situ* hybridisation are bifidobacteria[28] and they still make up 3%-5% of the adult microflora[29]. Thus, bifidobacteria undoubtedly constitute one of the predominant species of the human colonic microflora[30]. The analysis and annotation of the genome sequence of *B. longum* NCC2705 illustrated the close relationship of this bacterium with its human host[31]. Several beneficial health effects have been claimed to be related to the presence of bifidobacteria in the colon[32-41]. Based on these properties, bifidobacteria have become increasingly interesting for probiotic use, both in pharmaceutical application and dairy products[42]. One of these applications is their use in probiotic intervention in chronic intestinal inflammation. Probiotics containing bifidobacteria have been shown to be effective in reducing the severity of inflammation in several rodent models and patients with IBD[38,43-47].

In this study, the effects of a selection of bifidobacteria on IECs were investigated using an *in vitro* approach mimicking the situation of increased responsiveness of IECs to LPS. In this model, the antagonistic potential of several bifidobacteria on LPS-induced inflammatory responses in IECs was shown to be mediated by blocking NF-κB activation. This suggests that several *Bifidobacterium* strains, especially of *B. bifidum*, might be good candidates for probiotic intervention in IBD.

MATERIALS AND METHODS

Bacterial strains, cell lines and culture conditions

All bacterial strains used are shown in Table 1. For experiments, bacteria were cultured for 16 h at 37℃ (bifidobacteria: de Man-Rogosa Sharpe medium with 0.5 g/L cysteine, anaerobic; *E. coli*: brain heart infusion medium, aerobic; all media BD Difco, Basel, Switzerland). HT-29 cells were maintained at 50% CO_2 in DMEM (4.5 g/L glucose; Amimed, Basel, Switzerland) supplemented with 100 mL/L fetal calf serum (FCS; Amimed), 10 mL/L non-essential amino acids (NEAA, Sigma, Basel, Switzerland), 10 mL/L penicillin-streptomycin solution (pen-strep, Sigma). HT-29 clone 34 cells were obtained by stable transfection of the HT-29 cell line with the pNF-κB-SEAP-NPT plasmid[48]. This plasmid harbours the reporter gene for secreted alkaline phosphatase (SEAP). It contains

Table 1 Bacterial strains used in this study

Strain or oligonucleotides	Relevant characteristics	Source or PCR product
E. coli		
TG1	Cloning host	DSMZ[1]
D2241	Non-pathogenic intestinal isolate, control	NCC[2]
Bifidobacterium		
NCC362	*B. lactis, type strain*	NCC[2]
NCC2705	*B. longum, type strain*	NCC[2]
NCC251	*B. adolescentis, type strain*	NCC[2]
NCC189	*B. bifidum*	NCC[2]
S16	*B. bifidum, intestinal isolate from a breast-fed infant*	[57]
S17	*B. bifidum, intestinal isolate from a breast-fed infant*	[57]
E18	*B. infantis/longum, intestinal isolate from an adult*	[57]
MB226	*B. breve, type strain*	D. Matteuzzi

[1] DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen; [2] NCC: Nestlé Culture Collection.

the κB4 enhancer element fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter, which permits the expression of the reporter gene following activation of the NF-κB signalling pathway. The SEAP coding sequence is followed by the SV40 late polyadenylation signal to ensure proper and efficient processing of the SEAP transcript in eukaryotic cells. The vector backbone also contains a pUC origin of replication and an ampicillin resistance gene for propagation and selection in *E. coli*. In addition, it contains the neomycin phosphotransferase gene for the dominant selection marker of geneticin resistance in eukaryotic cells. After amplification in *E. coli* TG1, the plasmid was transfected into HT-29 cells using lipofectamine (Invitrogen, Basel, Switzerland) as described by Moon *et al*[48], and the stable clone 34, showing good kinetics of reporter gene activation using recombinant human TNF-α (10 μg/L, R&D Systems, Oxon, England) as a stimulus, was used for reporter gene assays. HT-29 clone 34 cells were cultured in DMEM with 100 mL/L FCS, 10 mL/L NEAA, and geneticin (0.5 g/L; Invitrogen). For experiments, both HT-29 and HT-29 clone 34 cells were seeded at 2×10^5 cells/mL in 12-well format cell culture plates (Falcon®, Milian, Geneva, Switzerland) and cultured in the respective medium until they reached 90%-100% confluence. At this stage, approximately 1×10^6 cells/well were counted.

LPS and TNF-α challenge of IEC lines

HT-29 or HT-29 clone 34 cells were grown as described above. At 90%-100% confluence, cells were washed and the medium was changed to 0.9 mL of DMEM with 100 mL/L FCS, 10 mL/L NEAA, 50 mmol/L Hepes (Invirogen), and penicillin G (100 kU/L; Fluka). Bacteria were grown as described above and added at the indicated multiplicity of infection (*moi*) in 0.1 mL of cell culture medium. In some experiments, cells were pre-incubated for 1 h with bacteria and subsequently stimulated with TNF-α (10 μg/L) or LPS (10 μg/L, serotype B55:O5; Sigma). Where indicated, a challenge was performed in the presence of 50 mL/L of human milk (HM). Milk was

sampled from different mothers (d 20 of lactation) and pooled. The pool of HM used for this study contained 26 mg/L of sCD14 (determined by ELISA; P. Serrant, personal communication). After 16 h of incubation, supernatants were collected by centrifugation and used for quantification of SEAP or IL-8. Viability of the eukaryotic cells was continuously checked by microscopic examination of trypan blue (Sigma) exclusion and was > 95% in all experiments.

Detection of SEAP and IL-8 protein

Phosphatase activity of SEAP was quantified by bioluminescence using the PhosphaLight™ kit (Tropix, MA, USA). Bioluminescence was measured as relative light units (RLU) in a TECAN SPECTRAFluor Plus spectrometer with an integration time of 100 milliseconds and gain set to 100. Data were analysed using the XFlour software version 4.40. RLU varied between experiments due to the nature of the assay. Where more than one experiment was used to present figures and for statistical analysis, correction for these day to day variations was performed by normalizing data to set 100 RLU units for the positive control (LPS + HM). In one experiment, levels of NF-κB activation were similar for 10 μg/L of TNF-α and 10 μg/L of LPS in combination with HM.

Secreted IL-8 protein was quantified by ELISA using the IL-8 Eli-pair kit (Diaclone, CT, USA). Absorbance was measured in a Dynex MRX microplate reader (Dynex Technologies, Worthing, England) at 450 nm.

Real-time PCR

For real-time PCR, RNA of HT-29 cells was isolated after bacterial pre-treatment and 4 h of LPS challenge in the presence of 5% (v/v) HM using the NucleoSpin® RNAII kit (Macherey-Nagel, Düren, Germany). RNA was quantified using Ribogreen® RNA quantification kit (Molecular Probes, Basel, Switzerland). Quality of RNA was verified using Agilent RNA 6000 Nano Assays and the 2100 Bioanalyzer (Agilent Technologies, CA, USA) with corresponding software (version A.01.16). 1 μg of total RNA was transcribed to cDNA using TaqMan Reverse Transcription Reagents. Real time reactions were set up using 5 μL TaqMan® 2 x PCR Master Mix, 0.5 μL of TaqMan® assays-on-demand primer/probe mixture (glyceraldehyde-3 phosphate dehydrogenase (GAPDH): Hs99999905_mL; IL-8: Hs00174103_mL; TNF-α: Hs00174128_mL; COX-2: Hs00153133_mL; ICAM-1: Hs00164932_mL; all reagents were purchased from Applied Biosystems) and 4.5 μL H_2O containing cDNA corresponding to 10-20 ng RNA, depending on the target gene. Real-time PCR was performed in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and analysed with the SDS software package version 2.1. Results of the different RNA samples were normalised for RNA quantity using the gene for GAPDH.

RESULTS

Bifidobacteria do not induce NF-κB-dependent reporter gene activity in HT-29 clone 34 cells

To study the effect of bifidobacteria on inflammatory events in IECs, i.e. on NF-κB activation, a reporter gene assay in HT-29 clone 34 cells was used. Cells were incubated with bacteria (*moi* = 100) for 16 h. Subsequently, activity of the NF-κB-driven reporter protein SEAP was measured in culture supernatants (Figure 1). While non-pathogenic *E. coli* D2241 induced significant reporter gene activity, all bifidobacteria tested failed to induce any activation of NF-κB above background levels. The same cell-based assay was used to investigate the effect of bifidobacteria on LPS-induced inflammation in IECs. HT-29 clone 34 cells were challenged with LPS for 16 h and subsequently SEAP activity was measured in the culture supernatants (Table 2). Only a 2.5-fold induction of NF-κB activation by LPS alone compared to un-stimulated cells was observed. To mimic the situation in the intestinal epithelium of IBD patients where expression of CD14 is increased, 50 mL/L of HM, containing sCD14, were added to the assay. Addition of HM led to a 50-fold increase in LPS-induced NF-κB activation, whereas HM alone did not result in activation of NF-κB. Also, in the presence of HM none of the tested bifidobacteria induced NF-κB-dependent reporter gene activity above background levels while stimulation with *E. coli* D2241 was enhanced about 200-fold (data not shown), further supporting the results indicating that bifidobacteria do not induce inflammatory events in IECs.

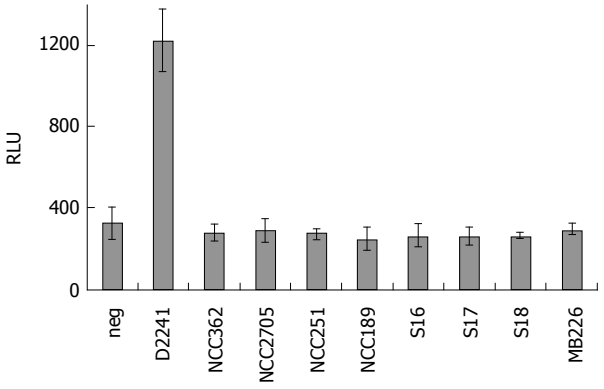


Figure 1 Quantification of NF-κB-driven SEAP activity in the supernatants of HT-29 clone 34 cells challenged with different bacterial strains at moi = 100 for 16 h. Results are shown as RLU and are mean ± standard deviation (SD) of triplicate measurements of one representative of three independent experiments. neg: negative control, no bacteria.

Table 2 Quantification of NF-κB-driven SEAP activity in the supernatants of HT-29 clone 34 cells

Treatment	RLU ± SD [1]
None (untreated control)	2 ± 1
HM	2 ± 1
LPS	5 ± 1
LPS + HM	100 ± 6

[1] Values are mean ± SD of at least 3 independent experiments for each condition performed in triplicate. For each individual experiment luminescence measurements were normalized to give 100 RLU for stimulation with LPS + HM.

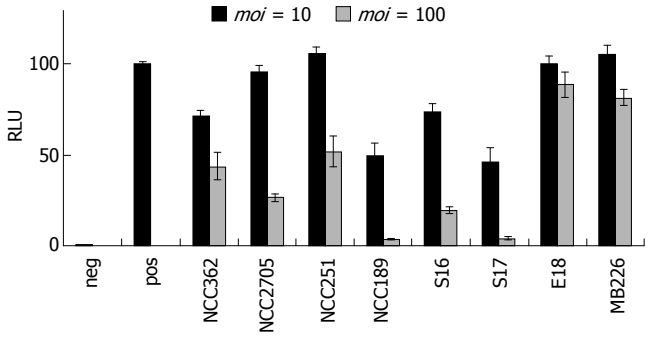


Figure 2 Dose- and strain-dependent inhibition of LPS-induced NF-κB activation by different bifidobacteria. SEAP activity was quantified in the supernatants of HT-29 clone 34 cells pre-incubated with different bifidobacteria at *moi* = 10 (dark grey bars) or *moi* = 100 (light grey bars) and subsequently stimulated with LPS + HM for 16 h. Cells treated with medium only served as negative control (neg; white bar) and positive controls (pos; black bar) were cells challenged with LPS + HM without bacterial pre-treatment. Results are means ± standard error of the mean (SEM) of three independent experiments performed in triplicate. RLU of indicidual experiments were normalised to give 100 RLU for the positive control.

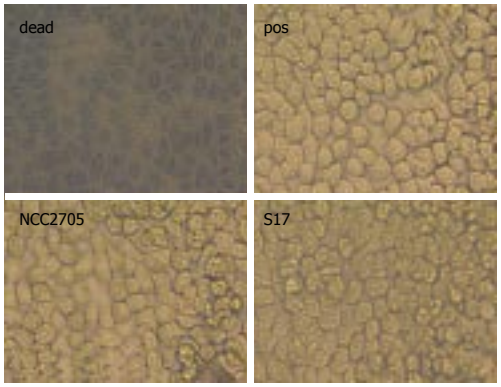


Figure 3 Microscopic examination of cellular viability. Trypan blue exclusion was monitored in HT-29 clone 34 cells challenged with LPS + HM for 16 after pre-incubation with bifidobacteria (*B. longum* NCC2705 or *B. bifidum* S17; *moi* = 100). Positive control (pos) was cells incubated with LPS + HM without bacterial pre-treatment. As a control for trypan blue staining cells were incubated for 16 h in cell culture medium at pH 4 (dead).

Strain- and dose-dependent inhibition of LPS-induced NF-κB activation by bifidobacteria

Several reports suggest anti-inflammatory effects of probiotics containing bifidobacteria[38,44,49]. Therefore, all bifidobacteria used in this study were assessed for their potential inhibitory effect on LPS-induced NF-κB activation at different bacterial doses. At a *moi* of 1, none of the bifidobacteria tested had an effect on NFκB-dependent reporter gene activity (data not shown). As shown in Figure 2, at a *moi* = 10, however, pre-treatment with *B. lactis* NCC362 and *B. bifidum* NCC189, S16, and S17 significantly decreased NF-κB-dependent reporter gene activity to 47%-71% of the positive control. When the bacterial dose for pre-treatment was further increased to 100 bacteria per cell (*moi* = 100), the inhibitory effect of *B. bifidum* NCC189 and that of *B. bifidum* S17 was almost complete (5% of positive control), while that of *B. lactis* NCC362, *B. longum* NCC2705, *B. adolescentis* NCC251 and *B. bifidum* S16 was intermediate (20%-52% of positive control). In contrast, *B. longum/infantis* E18 and *B. breve* MB226 had no or only very slight inhibitory effect on SEAP activation at either *moi*. To assess whether physical presence of bifidobacteria was required for their inhibitory effect, further experiments were carried out using cell culture medium pre-treated for 16 h with bifidobacteria followed by the removal of bifidobacterial cells using filter sterilization. Use of this conditioned medium did not result in any inhibition of LPS-induced NF-κB activation in HT-29 clone 34 cells (data not shown). These results show that six out of eight bifidobacteria tested significantly inhibited LPS-induced NF-κB activation in IECs and that this inhibitory capacity of bifidobacteria is strain- and dose-dependent. Furthermore, presence of bifidobacterial cells during LPS challenge is required for their anti-inflammatory effects indicating that this inhibition is not due to secreted compounds. To prove that decreased activity of the reporter protein was not a consequence of apoptosis due to high bacterial doses in combination with LPS, viability of the cells was confirmed by trypan blue exclusion. Microscopic examination of cells pre-treated with two representative strains (*B. longum* NCC2705 and *B. bifidum* S17), challenged with LPS + HM showed that viability was > 95% in all experiments demonstrating that inhibition of LPS-induced reporter gene activity by bifidobacteria is not due to cell death (Figure 3).

Table 3 IL-8 protein in the supernatants of HT-29 cells (mean ± SD, *n* = 3)

Treatment	IL-8 [μg/L] [1]	
	moi = 10	*moi* = 100
None (untreated control)	2 ± 1	
LPS+HM	36 ± 3	
NCC2705/LPS+HM 1	30 ± 2	7 ± 3
S17/LPS+HM 1	7 ± 2	2 ± 0

[1] Pre-incubation with *B. longum* NCC2705 or *B. bifidum* S17 at moi = 10 or 100 was followed by stimulation with LPS + HM for 16 h.

Inhibition of LPS-induced inflammatory events by B. bifidum S17 and B. longum NCC2705

To further investigate the effect of bifidobacteria on inflammatory events following NF-κB activation, IL-8 secretion and mRNA level of the genes encoding IL-8, TNF-α, COX-2, and ICAM-1 were monitored in normal HT-29 cells after bacterial pre-treatment with *B. bifidum* S17 and *B. longum* NCC2705 and a subsequent LPS challenge. Pre-incubation with *B. bifidum* S17 reduced IL-8 secretion of the IECs to about 20% of the positive control already at a *moi* = 10 and was able to completely inhibit IL-8 secretion at 100 bacteria per cell (Table 3). Effective inhibition with *B. longum* NCC2705 was only observed at a *moi* = 100. Thus, the dose-response of the IECs for IL-8 secretion was similar to that observed for NF-κB-driven SEAP reporter gene activity.

The effect of pre-treatment of HT-29 cells with bifidobacteria on mRNA levels for genes known to be regulated by NF-κB was investigated by real-time PCR. Pre-treatment with *B. longum* NCC2705 and *B. bifidum* S17 at a *moi* = 100 severely inhibited transcriptional activation of the genes for IL-8, TNF-α, COX-2, and ICAM-1 in

Table 4 Real-time quantification of mRNA levels of several genes in HT-29 cells

Treatment	Relative mRNA level [1] ± SD of gene for			
	IL-8	TNF-α	COX-2	ICAM-1
LPS+HM (positive control)	40.6 ± 17.4	37.4 ± 12.6	5.9 ± 1.7	28.0 ± 10.7
B. longum NCC2705/ LPS+HM [2]	11.4 ± 1.1[a]	12.7 ± 1.8[a]	2.5 ± 1.6	12.2 ± 9.2
B. bifidum S17/ LPS+HM [2]	3.5 ± 2.6[a]	2.9 ± 2.4[a]	1.4 ± 0.6[a]	2.0 ± 0.8[a]

[1] Results are mRNA levels relative to the negative control (no bacteria, no LPS) and are mean ± standard deviation (SD) of three independent pools of RNA for each condition. [2] Pre-incubation with *B. longum* NCC2705 or *B. bifidum* S17 at moi = 100 was followed by stimulation with LPS + HM for 4 h. [a] $P < 0.05$ *vs* positive control; Student's *t*-test.

HT-29 cells challenged with LPS in the presence of HM (Table 4). Again, *B. bifidum* S17 was more effective than *B. longum* NCC2705. Taken together, these results indicate that selected strains of bifidobacteria are able to inhibit LPS-induced inflammatory events in IECs.

TNF-α-induced NF-κB activation is not inhibited by bifidobacteria

NF-κB is activated through a wide range of stimuli other than LPS (see introduction). Therefore, it was interesting to test whether a similar inhibition of bifidobacteria could be observed for a pro-inflammatory stimulus other than LPS, such as TNF-α. At *moi* = 100, none of the tested bifidobacteria had any inhibitory effect on NF-κB-dependent reporter gene activity when 10 μg/L TNF-α was used to challenge HT-29 clone 34 cells (Figure 4). Further experiments were performed using a lower dose of TNF-α (1 μg/L) as stimulus and different doses of bacteria (*moi* = 10 and 100). In none of the tested conditions, an inhibitory effect of bifidobacteria on TNF-α induced NF-κB activation was observed (data not shown). These results suggest that the inhibitory capacity of bifidobacteria is specific for LPS-induced NF-κB activation in IECs.

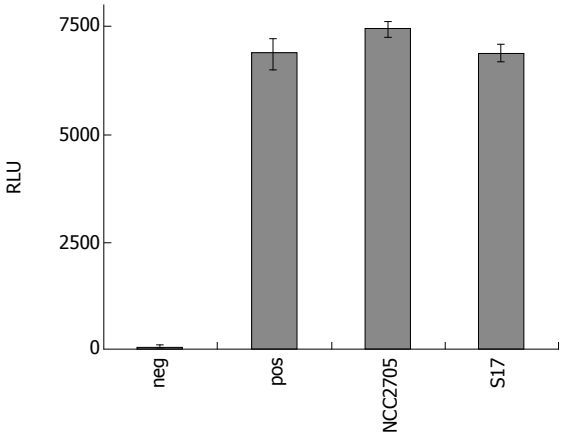


Figure 4 NF-κB-driven SEAP activity in the supernatants of HT-29 clone 34 cells pre-incubated with different bifidobacteria and subsequent stimulation with TNF-α (10 μg/L) for 16 h in the presence of 50 mL/L HM. Cells treated with medium only served as negative control (neg) and positive controls (pos) were cells challenged with TNF-α + HM without bacterial pre-treatment. Results are given as RLU and are means ± SD of triplicate measurements of a representative of two independent experiments.

DISCUSSION

Previous studies have shown that the intestinal microflora is implicated in the pathogenesis of IBD[2-4]. Increased expression of CD14 and TLR4 in IECs[19] might contribute to the sustained inflammation in the epithelium of patients of IBD. On the other hand, for several probiotic formulations, effects on inflammation in cultured IEC lines and in mouse models of chronic intestinal inflammation have been reported, yet there is a significant lack in our understanding of the molecular mechanisms by which probiotics downmodulate intestinal inflammation. Most studies on anti-inflammatory effects of probiotics were performed using mixtures of different probiotic strains, mainly lactobacilli and bifidobacteria[50]. For HT-29 and T84 cells, it was shown that incubation of these cells with VSL#3, a formulation containing eight different strains including 3 strains of bifidobacteria, resulted in decreased secretion of IL-8 in response to challenge with non-pathogenic *E. coli* or pathogenic *Salmonella dublin*[51]. In the IL-10 knock-out model of colitis, introduction of VSL#3 in the diet had an inhibitory effect on basal and LPS-stimulated TNF-α secretion in biopsies obtained from the colonic mucosa[44]. In the same mouse model, feeding a diet containing *B. infantis* 35624 was superior to *L. salivarius* UCC118 in effectively reducing the total gastrointestinal inflammatory score[38]. Finally, VSL#3 has been successfully used in the treatment of some IBDs[43,45].

Here, the effects of bifidobacteria on IECs were investigated with particular emphasis on LPS-induced inflammation. To our knowledge this represents the first attempt to characterize anti-inflammatory effects of whole cells of a single bifidobacterial strain. None of the tested bifidobacteria induced activation of NF-κB in HT-29 clone 34 cells, whereas a challenge with *E. coli* D2241 resulted in significant induction of NF-κB driven reporter gene activity in this cell line. Challenge of HT-29 clone 34 cells with LPS alone resulted in only a 2.5-fold induction of NF-κB-dependent reporter gene activity above background levels. This is consistent with results from other groups, which showed that colonic epithelial cells are poorly responsive to LPS[20]. However, using HM as a source of sCD14[52], the NF-κB response of HT-29 IECs to LPS was dramatically increased. This result supports previous studies showing increased responsiveness of HT-29 cells to LPS in the presence of HM[52-54]. This increased responsiveness of HT-29 cells to LPS in the presence of milk could be blocked by an anti-CD14 antibody[52].

NF-κB-dependent reporter gene activation by LPS in the presence of milk could be inhibited by pre-incubation with bifidobacteria in a strain- and dose-dependent manner, with the three *B. bifidum* strains (NCC189, S16, and S17) being most effective. To achieve this inhibitory effect, the presence of bifidobacterial cells was required, as filter-sterilized cell culture medium pre-treated with bifidobacteria did not show any inhibition of LPS-induced NF-κB activation. This indicates that the anti-

inflammatory compound is not actively secreted by bifidobacteria or released from lysed cells. Furthermore, incubation of HT-29 cells with *B. longum* NCC2705 and *B. bifidum* S17 prior to LPS challenge reduced IL-8 secretion and had a significant inhibitory effect on mRNA levels of the TNF-α, IL-8, ICAM-1, and COX-2 genes, all of which have been shown to be regulated by NF-κB[9]. In a similar study, VSL#3 inhibited NF-κB reporter gene activity and MCP-1 secretion after a challenge with TNF-α, in conditionally immortalised mouse colon cells[49]. In the presented experiments, none of the tested strains of bifidobacteria inhibited NF-κB activation in response to TNF-α. These results suggest that bifidobacteria interfere with pro-inflammatory signals upstream of the pathway of NF-κB activation common for LPS and TNF-α.

Lipoteichoic acids (LTA) of two lactobacilli strains were able to inhibit IL-8 secretion in response to LPS in HT-29 cells[54]. In a recent study, it was shown that the composition of LTA have an impact on the immunogenicity and anti-inflammatory capacity of *L. plantarum*[55]. Of note, a mutant lacking D-alanine substitutions to the back bone of LTA showed dramatically improved anti-inflammatory properties compared to the wild type. The mechanism by which bifidobacteria exert their inhibitory effect remains to be elucidated. One study investigating DNA from the VSL#3 mixture showed that the bifidobacterial strains' DNA was effective in limiting epithelial pro-inflammatory responses in IL-10-deficient mice and in HT-29 cells challenged with TNF-α. However, the pro-inflammatory response to LPS in HT-29 cells was not inhibited by the probiotic DNA[56]. This rules out, to a certain degree, that the effects observed in the presented experiments stem from DNA leaking from lysed bifidobacteria. Furthermore, it suggests that the mechanisms by which whole cells of bifidobacteria inhibit inflammatory responses are distinct from those described for their DNA.

Although anti-inflammatory activity of probiotics has been reported previously, to our knowledge, this is the first report on a specific inhibitory effect of bifidobacteria on LPS-induced inflammatory events in IECs, suggesting a role for bifidobacteria in down-modulation of pro-inflammatory cytokines[57]. Blocking of LPS-induced NF-κB activation by bifidobacteria could, at least in part, explain their positive effects on chronic intestinal inflammation *in vivo* through prevention of further amplification of the pro-inflammatory signal after exposure to LPS. Our results suggest that the capacity to inhibit LPS-induced NF-κB activation is strain-dependent. Especially, the strains of *B. bifidum* are promising candidates for probiotic intervention in inflammatory disorders of the gastrointestinal tract.

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BASIC RESEARCH

Sclerosing cholecystitis associated with autoimmune pancreatitis

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Abstract

AIM: To evaluate the histopathological and radiological findings of the gallbladder in patients with autoimmune pancreatitis (AIP).

METHODS: The radiological findings of the gallbladder of 19 AIP patients were retrospectively reviewed. Resected gallbladders of 8 AIP patients were examined histologically and were immunostained with anti-IgG4 antibody. Controls consisted of gallbladders resected for symptomatic gallstones (n = 10) and those removed during pancreatoduodenectomy for pancreatic carcinoma (n = 10), as well as extrahepatic bile ducts and pancreases removed by pancreatoduodenectomy for pancreatic carcinoma (n = 10).

RESULTS: Thickening of the gallbladder wall was detected by ultrasound and/or computed tomography in 10 patients with AIP (3 severe and 7 moderate); in these patients severe stenosis of the extrahepatic bile duct was also noted. Histologically, thickening of the gallbladder was detected in 6 of 8 (75%) patients with AIP; 4 cases had transmural lymphoplasmacytic infiltration with fibrosis, and 2 cases had mucosal-based lymphoplasmacytic infiltration. Considerable transmural thickening of the extrahepatic bile duct wall with dense fibrosis and diffuse lymphoplasmacytic infiltration was detected in 7 patients. Immunohistochemically, severe or moderate infiltration of IgG4-positive plasma cells was detected in the gallbladder, bile duct, and pancreas of all 8 patients, but was not detected in controls.

CONCLUSION: Gallbladder wall thickening with fibrosis and abundant infiltration of IgG4-positive plasma cells is frequently detected in patients with AIP. We propose the use of a new term, sclerosing cholecystitis, for these cases that are induced by the same mechanism as sclerosing pancreatitis or sclerosing cholangitis in AIP.

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Key words: Autoimmune pancreatitis; Sclerosing cholecystitis; IgG4

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a unique form of chronic pancreatitis characterized clinically and morphologically by irregular narrowing of the main pancreatic duct, enlargement of the pancreas, increased levels of serum γglobulin, IgG, or IgG4, the presence of autoantibodies, and responsiveness to steroid therapy. Patients with AIP frequently show stenosis of the bile duct system, including the intrahepatic and the extrahepatic bile duct[1-3]. Ultrasonography (US) or endoscopic ultrasonography (EUS) studies have demonstrated the bile duct wall thickening in segments without abnormalities on cholangiography, as well as in stenotic portions[4]. The histopathological findings in the pancreas of AIP are fibrosis and lymphoplasmacytic infiltration with abundant IgG4-positive plasma cells. However, these characteristics are also detected in the bile duct wall of patients with AIP[5,6]. These findings suggest a close relationship between AIP and sclerosing cholangitis[7], though there are only a few studies that have evaluated the gallbladder in AIP[8]. In the present study, we evaluated the histopathological and radiological findings of the gallbladders of AIP patients.



Figure 1 Severe thickening of the gallbladder wall in a patient with autoimmune pancreatitis on US.



Figure 2 Moderate thickening of the gallbladder wall in a patient with autoimmune pancreatitis on US.



Figure 3 Transmural lymphoplasmacytic infiltration with fibrosis in the gallbladder wall of a patient with autoimmune pancreatitis.

MATERIALS AND METHODS

Patients

The study subjects were 19 patients with AIP in whom the gallbladder was examined by US and computed tomography (CT) before treatment for AIP. The patients were diagnosed based on the following criteria: (1) irregular narrowing of the main pancreatic duct (n = 19); (2) enlargement of the pancreas (n = 17); (3) pancreatic histology showing dense lymphoplasmacytic infiltration with fibrosis (n = 8); (4) elevation of serum γglobulin (≥ 2.0 g/dL, n = 13), IgG (≥ 1800 mg/dL, n = 13), IgG4 (≥ 136 mg/dL, n = 14); (5) presence of autoantibodies (n = 9); and (6) morphological and serological effectiveness of steroid treatment (n = 11). A clinical diagnosis of AIP was made in all patients who in addition to fulfilling criteria 1, met at least 2 of the remaining criteria.

Six patients had a pancreatoduodenectomy, and 2 had a bile duct resection, cholecystectomy, and a choledochoduodenostomy with a pancreatic biopsy. These 8 patients had obstructive jaundice due to marked stenosis of the extrahepatic bile duct and were suspected of having carcinoma of the head of the pancreas. Stenosis of the extrahepatic bile duct occurred in 16 patients (1 upper bile duct and 15 lower bile ducts).

Methods

The radiological findings of the gallbladders of all 19 patients were retrospectively reviewed. The degree of thickening of the gallbladder wall was classified into 3 categories: severe (thickness ≥ 8 mm, Figure 1); moderate (thickness from ≥ 4 mm to < 8 mm, Figure 2); and mild or none (thickness < 4 mm).

At least 3 sections of the resected gallbladder of the 8 AIP patients were histologically examined and immunostained by anti-IgG4 antibody (The Binding Site, Birmingham, UK) with avidin-biotin-peroxidase complex (ABC). The pattern of gallbladder inflammation was classified into 2 types: transmural in which the inflammatory infiltrates extended through the gallbladder wall and included involvement and focal destruction of the muscularis (Figure 3); and mucosal-based, in which the inflammatory infiltrates were prominently detected within the lamina propria. The degree of histological thickening of the bile duct wall was classified into 3 categories: severe (thickness ≥ 4 mm); mild (thickness from ≥ 2 mm to < 4 mm); and none (thickness < 2 mm).

The number of immunohistochemically identified cells per high power field (HPF) in each specimen was counted. The degree of infiltrated IgG4-positive plasma cells was classified as severe (>20/HPF, Figure 4); moderate (10-20/HPF); mild (5-9/HPF); and few (0-4/HPF). The gallbladders resected for symptomatic gallstones (n = 10), those removed during pancreatoduodenectomy for pancreatic carcinoma (n = 10), and the extrahepatic bile ducts and pancreases removed during pancreatoduodenectomy for pancreatic carcinoma (n = 10) were also examined histologically and immunohistochemically as controls.

RESULTS

Thickening of the gallbladder wall was detected on US and/or CT in 10 patients with AIP [3 severe (Figure 1) and 7 moderate (Figure 2)]. All of the 10 patients also had stenosis of the extrahepatic bile duct. The presence of gallstones was noted in only 2 patients.

Histologically, thickening of the gallbladder was

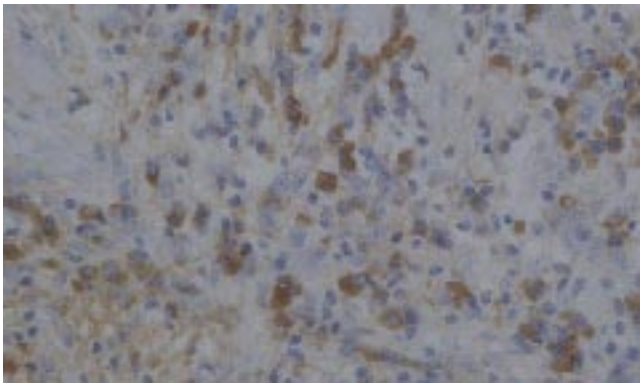


Figure 4 Severe infiltration of IgG4-positive plasma cells in the gallbladder wall of a patient with autoimmune pancreatitis.

Table 1 Relationship between thickening of the gallbladder wall and infiltration of IgG4-positive plasma cells in the gallbladder wall, bile duct wall and pancreas

Thickening of the gallbladder wall *n*	Infiltration of IgG4-positive plasma cells					
	Gallbladder wall		Bile duct wall		Pancreas	
	Severe	Moderate	Severe	Moderate	Severe	Moderate
Transmural: severe 4	4	0	4	0	4	0
Mucosal-based 2	2	0	2	0	2	0
None 2	0	2	1	1	2	0

detected in 6 of 8 (75%) patients with AIP who underwent surgery; 4 of these 6 patients had transmural lymphoplasmacytic infiltration with fibrosis (Figure 3), and 2 had mucosal-based lymphoplasmacytic infiltration. Lymphoid nodule formation was detected in 2 patients. No patients had dysplastic or neoplastic changes of the gallbladder epithelium. Considerable transmural thickening of the extrahepatic bile duct wall with dense fibrosis and diffuse lymphoplasmacytic infiltration was detected in 7 patients. However, transmural thickening of the gallbladder wall with lymphoplasmacytic infiltration and fibrosis was not detected in the controls.

Immunohistochemically, severe infiltration of IgG4-positive plasma cells was detected in the pancreas of all patients. Severe infiltration of IgG4-positive plasma cells was also detected in the gallbladder (Figure 4) and bile duct wall of 6 patients with thickening of the gallbladder wall. Moderate infiltration of IgG4-positive plasma cells was also detected in the non-thickened gallbladders. In all control cases, IgG4-positive plasma cell infiltration in the gallbladder, bile duct, and pancreas was classified as few (Table 1).

DISCUSSION

In AIP, stenosis of the bile duct system is detected from 94%[9] to 100%[10] of patients. In most patients, the lower bile duct is narrowed, and the intrahepatic bile duct or upper bile duct is occasionally involved. Histologically, dense lymphoplasmacytic infiltration with fibrosis, which is the characteristic finding in the pancreas of AIP patients, is also detected in the transmural bile duct wall of many patients with AIP[5,6]. Immunohistochemically, abundant infiltration of IgG4-positive plasma cells is specifically detected in the pancreas of AIP, but the infiltration is also detected in the bile duct wall of AIP patients[5,6]. Bile duct involvement in AIP is considered to be sclerosing cholangitis, which is induced by the same mechanism as sclerosing pancreatitis[7].

According to a report[8] on the pathology of gallbladder lesions in patients with AIP, 12 of 20 (60%) cases of AIP showed intense inflammatory infiltrates, and 7 cases (35%) showed transmural chronic cholecystitis. In the present study, severe or moderate thickening of the gallbladder wall was radiologically detected in 10 of 19 (53%) AIP patients; in these 10 patients, stenosis of the extrahepatic bile duct was also observed. Histologically, thickening of the gallbladder wall was detected in 6 of 8 (75%) AIP patients who underwent surgery; 4 of the 6 showed transmural inflammation and 2 showed mucosal-based inflammation. These 6 patients also showed severe transmural thickening of the extrahepatic bile duct wall. Immunohistochemically, severe or moderate infiltration of IgG4-positive plasma cells was detected in the gallbladder, bile duct, and pancreas of all 8 AIP patients in whom surgery was done, but was not detected in controls. These findings suggest that thickening of the gallbladder wall is one of the extrapancreatic lesions that is frequently detected in AIP patients and is characterized by transmural wall thickening with abundant infiltration of IgG4-positive plasma cells. Thus, we propose the use of a new term, sclerosing cholecystitis, to describe this condition that is induced by the same mechanism as sclerosing pancreatitis or sclerosing cholangitis in AIP.

In conclusion, sclerosing cholangitis is one of the extrapancreatic lesions that is frequently detected in AIP patients and is characterized by transmural wall thickening with abundant infiltration of IgG4-positive plasma cells.

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BASIC RESEARCH

Immunohistochemical expression of mismatch repair genes: A screening tool for predicting mutator phenotype in liver fluke infection-associated intrahepatic cholangiocarcinoma

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Abstract

AIM: To clarify possible contributions of DNA mismatch repair (MMR) system in carcinogenesis of liver fluke infection-associated intrahepatic cholangiocarcinoma (ICC) by using immunohistochemical assay.

METHODS: A total of 29 ICC samples, which had been assessed for genomic instability by a PCR-based method, were used for study. They were examined immunohistochemically to demonstrate protein expression of two MMR genes, hMSH2 and hMLH1. Results obtained were compared with their mutator phenotype assessed previously.

RESULTS: Either hMSH2 or hMLH1 protein was obviously expressed in 28 of 29 (96.6%) ICC samples. Positive nuclear localization of hMSH2 or hMLH1 protein was observed in 86.2% (25/29) or 93.1% (27/29) ICC cases, respectively, while their negative nuclear reactivity was only detected in 13.8% (4/29) or 6.9% (2/29) ICC cases analyzed, respectively.

CONCLUSION: Our study, probably for the first time, showed through immunohistochemical detection of hMSH2 and hMLH1 gene that DNA MMR system does not play a prominent role in liver fluke infection-associated cholangiocarcinogenesis. These results confirm previous findings on mutational status of these genes assessed through a PCR-based method. The immunohistochemical analysis has proven to be an effective and sensitive approach for screening MMR deficiency regardless of somatic inactivation or promoter hypermethylation of hMSH2 and/or hMLH1 gene. Furthermore, immunohistochemistry is more advantageous compared to mutator phenotyping assay in terms of simplicity, less time consuming and cost effectiveness for screening possible involvements of target MMR genes in tumorigenesis.

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Key words: Liver fluke infection; Cholangiocarcinoma; Mismatch repair; hMSH2; hMLH1; Immunohistochemistry; MSI; Mutator phenotype

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INTRODUCTION

The multistep process of carcinogenesis results from multiple genetic changes in oncogene(s), tumor suppressor gene(s) and/or DNA integrity gene(s)[1,2]. hMSH2 and hMLH1, the common human mutS homolog 2 and mutL homolog 1, respectively, are two key genes implicated in DNA mismatch repair (MMR) machinery[3]. They are among other stability genes that function as DNA mismatch restorers for the maintenance of genomic integrity[2]. Defects in this repair system are involved in the failure to recognize and/or repair spontaneous errors during the replication process. MMR deficiency leads to the accumulation of secondary mutations resulting from base-base mismatches and/or short insertion or deletion mispairings throughout the genome; this deficiency affects important growth regulatory genes and thereby leads to carcinogenesis[4]. Most cells that are deficient in this repair

system often display a high level of microsatellite instability (MSI), which generates a mutator phenotype, a hallmark of human cancers, as a consequence of DNA replication errors and homologous recombination[5,7]. Defects in MMR genes have recently been described as an alternative pathway in the pathogenesis of and predisposition toward a significant proportion of certain inherited and sporadic human malignancies[8-11].

Intrahepatic cholangiocarcinoma (ICC), a primary adenocarcinoma and the second most common cancer of the liver, develops from the overgrowth of cholangiocytes, which are epithelial cells that line intrahepatic ducts. Patients with this disease have a poor prognosis: ICC is usually fatal because of the difficulty in detecting it at an early stage[12,13]. Based on an epidemiological study, the incidence of ICC is dependent on geography[14]. This disease is most common in Southeast Asia[15], especially Thailand[15-16], and its etiology is thought to be multifactorial. Documented risk factors include sclerosing cholangitis, hepatolithiasis, congenital cysts and, especially, liver fluke infection[17]. Furthermore, regular alcohol consumption has also recently been found to be one of the risk factors for this tumor[18]. Known predisposing conditions, such as mechanical irritation and chronic inflammation, exert marked and long-term effects on the development and progression of this tumor by inducing epithelial proliferation and promoting the subsequent susceptibility of these tissues to exogenous and/or endogenous carcinogenic exposure, leading to cholangi ocarcinogenesis[19]. In Thailand, ICC is one of the most common cancers and lethal diseases in the North and Northeast regions[20]. In Khon Kaen province, where liver fluke (*Opisthorchis viverrini*) infection is prevalent[20,21], 90% of the local inhabitants are or have been infected with this parasite[22]. In this same region, ICC occurs at its highest rate worldwide[16,18,21,23].

Although the roles of hMSH2 and hMLH1 genes in the pathogenesis of colorectal cancer (CRC), especially the hereditary nonpolyposis colon cancer (HNPCC), have been described[24], little is known about their involvement in cholangiocarcinogenesis. An analysis of the mutational status of the hMSH2 and hMLH1 genes in ICC from Thai patients was carried out via assessments of genomic instability in repeat sequences of microsatellites. The low frequency of somatic mutations in hMSH2 and hMLH1 genes indicated a minor involvement of this repair system[25]. However, MMR gene inactivation is not always caused by somatic mutations, but rather can be achieved by hypermethylation at the gene promoter, as reported in the hMLH1 gene[26,27]. Currently, the identification of a mutator phenotype in tumors still requires molecular testing. As an alternative screening tool for detecting DNA MMR deficiency derived from either somatic mutations or promoter hypermethylation of target MMR genes, we used immunohistochemical staining for cellular expression of hMSH2 or hMLH1 protein in archival tissues from Thai ICC that had previously been assessed for mutator phenotype by mutational inactivation using the PCR-based method. Nuclear localization of encoded proteins evaluated from the current study and the mutator phenotype assessed from the previous investigation were comparatively analyzed.

MATERIALS AND METHODS

Tissue samples

A total of 29 cases consisting of 11 fresh-frozen and 18 formalin-fixed, paraffin-embedded ICC tissues were used for this study. Samples were obtained with informed consent and the project was approved by the Ethical Committee of the National Cancer Institute of Thailand. After surgery, parts of specimens were promptly fixed in neutral formalin and processed histologically to prepare tissue sections for further immunohistochemical analysis.

Immunohistochemical study

Immunohistochemical staining for hMSH2 and hMLH1 proteins was performed with a standard avidin-biotin-peroxidase complex technique[28], using diaminobenzidine (DAB) as a chromogen. Formalin-fixed, paraffin-embedded tissue sections (4-μm thick) of the ICC were processed immunohistochemically. Tissue sections were first dipped in three changes of xylene for 5 min each and three changes of absolute ethanol for 5 min each, followed by two washes with distilled water and a final wash in PBS for 5 min. Antigen retrieval was performed by immersing tissue sections in sodium citrate buffer (0.01 mol/L, pH 6.0), autoclaving for 10 min at 121℃, cooling and rewashing the sections in distilled water prior to incubating for 15 min in 30 mL/L H_2O_2 in methanol to block endogenous peroxidase activity. To eliminate non-specific protein binding, after three washes with PBS for 3 min each, tissue sections were incubated at room temperature for 10 min with 100 mL/L normal goat serum in PBS. Immunostaining was performed by incubating tissues with a primary antibody against hMSH2 protein (clone FE11, Oncogene Research, 1:50 dilution), hMLH1 protein (clone 14, Oncogene Research, 1:10 dilution) or PBS (as a negative control) in a humidified chamber at 4℃ overnight. Tissue sections were washed three times with PBS, 3 min each, followed by incubation with biotinylated rabbit anti–mouse IgG (1:1000 dilution) for 30 min at room temperature. After three washes for 3 min each in PBS, sections were incubated in a streptavidin-peroxidase reagent for another 30 min. After three PBS washes, the peroxidase reaction was initiated by incubating tissue sections in DAB reagent for 5-20 min at room temperature. Sections were washed three times in distilled water for 3 min each. Immunostained tissue sections were then lightly counterstained with Mayer hematoxylin, washed with distilled water, dehydrated with ascending graded ethanol and xylene and mounted with mounting medium for further viewing under the light microscope.

The expression of hMSH2 or hMLH1 protein is normally observed in the nucleus. A case was considered immunoreactively positive or negative when nuclear staining for either target protein was present in or completely absent from tumor cells in ICC tissues, respectively.

Assessment of mutator phenotype

MS alterations were previously assessed in each normal DNA and paired tumor DNA in the majority of this same set of samples (except cases 26 and 27) using

Table 1 Immunohistochemical expression of hMSH2 and hMLH1 genes and microsatellite profile in ICC cases analyzed

Case	Age/sex	Histology	pTMN stage	MS phenotype	hMSH2	hMLH1
1	50/M	G3	IVA	o	+	+
2	60/F	G2	IIIB	o	+	+
3	60/F	G2	IVA	o	+	+
4	55/F	G2	IVB	MSI (D17S250)	+	+
5	57/M	G3	IVA	o	+	+
6	71/M	G2	IIIB	o	+	+
7	65/F	G3	IIIA	MSI (D2S123)	-	-*
8	41/F	G1	II	MSI (D2S123)	-	+
9	60/M	G2	IIIB	o	-	+
10	57/M	G2	IVA	MSI (D17S250)	+	+
11	55/M	G2	U	o	+	+
12	55/M	GX	IVB	MSI (D2S123)	+	+
13	54/M	G1	IVA	o	+	+
14	59/F	G2	IVB	o	+	+
15	45/F	G3	IVB	o	+	+
16	42/M	G2	IIIB	o	+	+
17	57/M	G1	IIIA	MSI (D2S123)	-	+
18	51/F	GX	IVB	MSI (BAT-25)	+	+
19	42/M	G2	II	o	+	-
20	51/M	G3	IIIA	o	+	+
21	59/M	G3	IVA	MSI (D2S123)	+	+
22	66/M	G3	IVB	o	+	+
23	64/F	G3	IVA	o	+	+
24	66/M	G1	II	o	+	+
25	42/M	G2	IVB	o	+	+
26	37/F	G3	II	N	+	+
27	57/M	G1	III	N	+	+
28	66/M	G2	IVB	o	+	+
29	52/M	G3	IVB	o	+	+

G1: well differentiated; G2: moderately differentiated; G3: poorly differentiated; GX: grade cannot be assessed; U: not known; M: male; F: female; +: positive nuclear staining; -: negative nuclear staining; MSI: microsatellite instability; o: microsatellite stable; N: MSI status was not available; *: harbouring LOH at D3S1298 and D3S1561.

a panel of 12 known highly polymorphic MS loci in nuclear DNA: five Bethesda markers including BAT-25, BAT-26, D2S123, D5S346 and D17S250; and seven other markers including D2S119, D3S1277, D3S1298, D3S1561, D3S1611, D11S904 and TP53. Three different markers of repeat sequences in mitochondrial DNA, including the D-loop, NADH dehydrogenase subunit 1 and NADH dehydrogenase subunit 5, were also analyzed using a fluorescence PCR–based technique, as reported previously[25].

RESULTS

Immunohistochemical study

The immunohistochemical analysis for nuclear localization of two MMR genes, hMSH2 and hMLH1, in 29 ICC tissues demonstrated positive immunostaining in the majority of stained cases (Table 1). Intense nuclear expression of either hMSH2 or hMLH1 was observed in 96.6% (28/29) ICC cases. hMSH2 nuclear staining was readily visible in 86.2% (25/29) ICC cases (Figure 1 A and B), whereas positive immunostaining for the hMLH1 protein was detected in 93.1% (27/29) cases (Figure 1, C and D). Consequently, negative immunoreactivities for nuclear expression of hMSH2 and hMLH1 genes were verified in 4 (13.8%, cases 7, 8, 9 and 17) and 2 (6.9%, cases 7 and 19) of the 29 ICC cases, respectively (Table 1).

Among positively stained cases, different levels of expression of either MMR gene were noted. An intense immunohistochemical reaction was observed in cases 2, 12, 14 and 16 (16.0%) for hMSH2 and cases 18, 26, 27 and 29 (14.8%) for hMLH1. A relatively reduced degree of nuclear expression was observed in cases 1, 4, 5, 10 and 15 (20.0%) for hMSH2 and cases 4, 5, 10 and 21 (14.8%) for hMLH1.

Table 2 Correlation between hMSH2 and hMLH1 immunohistochemical expression and microsatellite profile in ICC studied *n*(%)

MS status	Number of cases	Pattern of nuclear expression/*n* (%)			
		hMSH2+ hMLH1-	hMSH2- hMLH1+	hMSH2+ hMLH1+	hMSH2- hMLH1-
MSI	8	0 (0)	2 (25)	5 (62.5)	1 (12.5)
MSS	19	1 (5.3)	1 (5.3)	17 (89.5)	0 (0)
NP	2	0 (0)	0 (0)	2 (100)	0 (0)
Total	29	1 (3.4)	3 (10.3)	24 (82.8)	1 (3.4)

MSI: Microsatellite instability; MSS: Microsatellite stable; NP: MSI assessment was not performed.

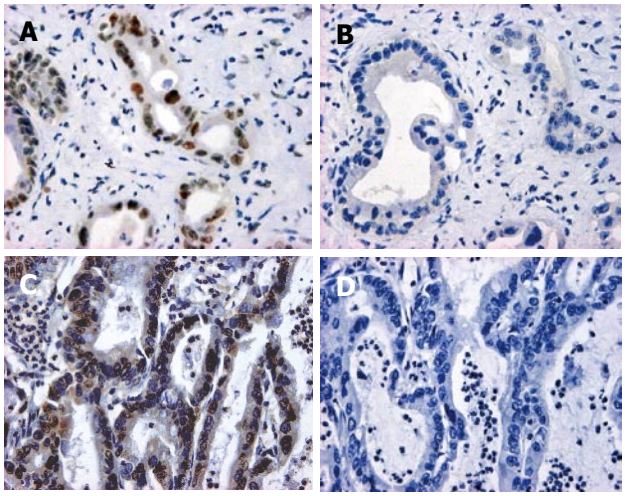


Figure 1 Representative data of the immunohistochemical expression of hMSH2 (case 26: A and B) or hMLH1 (case 22: **C** and **D**) protein in ICC. **A** and **C**: Stained with anti-hMSH2 and anti-hMLH1 antibodies, respectively; **B** and **D**: stained without the first antibodies as negative controls (immunoperoxidase, original magnification x 400).

Mutator phenotype study

Among the 15 microsatellite markers that were assessed, 8 loci were found to be aberrant. Low-frequency MSI was detected for BAT-25, D2S123, D11S904 and D17S250, and loss of heterozygosity (LOH) was detected for D3S1298, D3S1561, D5S346 and TP53. Most ICC samples were therefore classified as microsatellite stable (MSS), and a subset was classified as low-frequency MSI (MSI-L), as reported previously[25].

DISCUSSION

The assessment of MMR deficiencies has become an important tool for the characterization of genomic instability and of the molecular pathogenesis of both inherited and sporadic human cancers. Molecular defects in known MMR genes, especially hMSH2 and hMLH1, have been well defined in the HNPCC syndrome[29-31]. In subsets of sporadic human cancers, these two genes were also found to be involved in the pathogenesis with varying frequency[3]. Previous studies demonstrated that in a majority of sporadic MSI tumors, hMLH1 inactivation was frequently caused by an epigenetic rather than genetic mechanism, whereas the rest displayed somatic mutations in these two genes[32-34]. Methylation of the hMLH1 gene promoter is usually biallelic[35]. Assessments of microsatellite alterations are characterizations of a mutator phenotype through somatic mutational inactivation, however, do not identify aberrations in specific genes involved, which requires further investigation.

In ICC from Thai patients, hMSH2 and hMLH1 genes do make a minor contribution to cholangiocarcinogenesis, as shown by the assessment of the mutator phenotype through their somatic mutations, using a panel of highly polymorphic MS markers that are mainly related to these genes[25]. However, the inactivation of these genes via other genetic mechanisms has not yet been excluded. In the present study, using an immunohistochemical analysis, aberrations either through somatic mutations and/or promoter hypermethylation of hMSH2 and hMLH1 gene were sought at a protein level by evaluating the nuclear expression of their proteins in ICC carcinogenesis. Our results clearly showed immunoreactivity of either protein with different degree of expression level in the majority of cases, thereby indicating the minor involvement of these genes in the pathogenesis of this tumor (Table 1), which is consistent with results that were obtained through molecular assessments[25]. In a few cases of them, however, expression of one or both proteins was lost (Table 1). Considering the different degree of MMR gene expression noted in positively stained cases, a relatively lower degree of hMSH2 and hMLH1 immunostaining was observed in 20.0% (5/25) and 14.8% (4/27) immunopositive cases, respectively, giving rise to 21.4% (6/28) cases that presented with reduced expression of at least one MMR gene, suggesting that the down-regulation of the encoding genes occurred during their carcinogenic process. This phenomenon might have been due to hypermethylation at the gene promoter, at least in the case of the hMLH1 gene, of which was often accompanied by the down-regulation or absence of hMLH1 gene expression[34,36,37]. Hypermethylation-associated reduced expression of the hMLH1 gene has already been described in lung[38], gastric[39,40] and colorectal cancers[34,36].

There was a correlation between the nuclear expression of hMSH2 or hMLH1 protein and a previously assessed MSI/mutator phenotype in a given ICC tissue (Table 1). In hMSH2 immunostaining, 3 of 4 cases (cases 7, 8 and 17) that displayed negative nuclear staining also had MSI at D2S123, a hMSH2-related MS locus[25]. The remaining case (case 9), which also exhibited loss of hMSH2 gene expression, showed stability at this locus, suggesting the presence of other hMSH2-related MS loci that mapped to 2p is involved in hMSH2 somatic inactivation. Simultaneously, in hMLH1 immunostaining, two cases (cases 7 and 19) showed a loss of this gene expression. One of them (case 7) also exhibited LOH at two hMLH1-related MS loci, D3S1298 and D3S1561, whereas the other one was MSS[25] (Table 2), indicating an availability of other hMLH1-related loci that mapped to 3p, whose MSI/LOH are involved in the loss of expression of this gene.

In the current study, the majority of ICC samples (89.5%), almost all of which was previously assessed as MSS/MSI-L[25], consistently displayed positive nuclear staining for both MMR proteins (Table 2). Five (cases 4, 10, 12, 18, 21) of eight MSI cases (62.5%) retained both hMSH2 and hMLH1 protein expression (Tables 1 and 2). These results are in agreement with previous findings describing the presence of MSI tumors with positive immunohistochemical staining and without detectable somatic mutations in either MMR gene[41]. On the other hand, three (cases 1, 5, 15) of the MSS cases in this study had reduced expression of either or both MMR proteins. Nevertheless, previous findings demonstrated that relatively low level of hMSH2 and hMLH1 proteins may be sufficient to retain the MSS phenotype[42], which might also be the case for the current study. Certain incompatibilities between MSI and protein expression in cases 4, 10, 12, 18 and 21 might have been due to the existence of other as yet unknown stability gene(s) or another related gene(s) that function in or outside the DNA MMR complex and play a major role in the development of this tumor type. The present findings undoubtedly demonstrated and confirmed the previous findings that only a small fraction of ICC cases was affected by hMSH2 and/or hMLH1 functional inactivation and that these two MMR genes do not seem to be implicated in carcinogenesis steps in most ICC cases.

In conclusion, to our best of knowledge, we are the first to report the pattern of immunohistochemical expression of MMR genes in ICC. Results from previous mutator phenotype assays and the current immunohistochemical screening of hMSH2 and hMLH1 genes in ICC from Thai patients consistently confirm the minor involvement of the DNA MMR system, through malfunctional activity of the hMSH2 and/or hMLH1 genes, in ICC carcinogenesis. The immunohistochemical analysis has proven to be an effective and useful approach for the screening of defects in the DNA MMR complex derived from either somatic mutational inactivation or promoter hypermethylation of target MMR genes. It is more advantageous, in terms of simplicity, less time consuming and cost effectiveness, to screen mutator phenotype through inactivation of target MMR genes in cholangiocarcinogenesis by using the immunohistochemical method.

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RAPID COMMUNICATION

Significance of preoperative C-reactive protein as a parameter of the perioperative course and long-term prognosis in squamous cell carcinoma and adenocarcinoma of the oesophagus

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Abstract

AIM: C-reactive protein (CRP) is an acute-phase reactant and a known indicator of the malignant potential of the tumour. The aim of this study was to investigate the significance of preoperative CRP as a parameter of the perioperative course and long-term prognosis in patients with squamous cell carcinoma and adenocarcinoma of the oesophagus.

METHODS: Serum CRP was determined preoperatively in 291 of 371 patients undergoing oesophagectomy for cancer from December 1989 to March 2004. Median patient age was 59 (28-79) year, 82.5% of patients were males. Squamous cell carcinoma was diagnosed in 151 (51.9%) and adenocarcinoma in 122 patients. Transhiatal oesophagectomy was done in 151 (51.9%) patients and 134 (46.0%) patients underwent the abdominothoracic procedure.

RESULTS: In 127 (43.6%) patients the preoperative serum CRP concentration was within the normal range (< 5 mg/dL), elevated CRP levels were measured in 164 (56.4%) patients. Tumour extension (P < 0.0005) and the number of lymph nodes affected by metastatic spread (P = 0.015) were significantly increased in the group with elevated CRP levels. Among the perioperative parameters both the number of blood transfusions (P = 0.006) and the general complication rate (P = 0.002) were higher in patients with elevated preoperative CRP levels. The long-term survival rate of 13.6 (0-109.8) mo was poorer in the group with elevated CRP levels compared to 18.9 (0-155.4) mo in the group with normal CRP levels (log-rank test: P = 0.107). Multivariate analysis with backward variables selection identified preoperative CRP as an independent prognostic factor of the long-term prognosis in patients with oesophageal carcinoma, with a hazard ratio of 1.182 (95% confidence interval: 1.030-1.356).

CONCLUSION: The preoperative serum CRP-level is an easily determined independent prognostic marker in patients with squamous cell carcinoma and adenocarcinoma of the oesophagus.

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Key words: Preoperative C-reactive protein; Perioperative course; Long-term prognosis; Squamous cell carcinoma; Adenocarcinoma; Oesophagus

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INTRODUCTION

C-reactive protein (CRP) is an acute-phase reactant and a known indicator of the malignant potential of tumours. The synthesis is regulated by proinflammatory cytokines, which serve as growth factors in neoplastic processes[1]. These include in particular IL (interleukin)-1, IL-6, TNF (tumour necrosis factor)-α, IF (interferon)-γ, TGF (tumour growth factor)-β, EGF (epidermal growth factor) and LIF (leukaemia inhibiting factor). CRP is produced in the liver and electrophoresis shows a location of the protein between the beta- and gamma-globulin fractions.

The preoperative serum elevation of CRP has been identified as a significant prognostic factor in patients with colorectal cancer[2]. However, raised CRP concentrations as an indicator of a poorer prognosis in patients with oesophageal cancer have been demonstrated solely for squamous cell carcinoma[3,4]. The aim of this prospective study was to investigate the significance of C-reactive protein for the perioperative course and long-term

prognosis in a patient population with histologic entities of both adenocarcinoma and squamous cell carcinoma of the oesophagus.

MATERIALS AND METHODS

C-reactive protein was determined preoperatively in 291 of 371 patients undergoing oesophagectomy for carcinoma from December 1989 to March 2004. Median patient age was 59 (range from 28 to 79) year; 240 (82.5%) of the patients were males. Squamous cell carcinoma was diagnosed in 145 and adenocarcinoma in 122 patients. An undifferentiated carcinoma was found in 18 patients and 4 patients had other malignant tumours of the oesophagus. Patients receiving neoadjuvant chemoradiotherapy were excluded from the study.

Transhiatal oesophagectomy was carried out in 151 (51.9%) patients and 134 (46.0%) patients underwent abdominothoracic oesophagectomy with two-field lymphadenectomy (abdominal and mediastinal). Reconstruction was accomplished by pulled-up gastric tube in 264 of 285 patients (93.0%), by colon interposition in 12 (4.2%), and by small intestine interposition in 7 (2.5%) patients. The anatomic prevertebral oesophageal bed was used for the majority of these procedures. The remaining two patients did not undergo a primary reconstructive procedure.

Abdominothoracic oesophagectomy was routinely performed for squamous cell carcinoma. A transhiatal procedure was selected only for tumours with a distal location and malignancies without oesophageal wall penetration, or in the presence of a high general risk. Transhiatal oesophagectomy with abdominal and posterior mediastinal lymphadenectomy was carried out in adenocarcinomas, the two-field procedure was done in the presence of advanced tumour growth or extended lymph node involvement.

Data were collected prospectively in a specially established database and analysed retrospectively. In addition to routine demographic data, preoperative and intraoperative variables, tumour characteristics, as well as postoperative morbidity and mortality were documented. Long-term survival data were finally recorded in April 2004 and collected by telephone interview with the patients or their primary physicians.

CRP measurement

Serum CRP was determined by latex-enhanced homogeneous immunoassay[5] (wide-range) (Hitachi 917; Skill, Munich, Germany). In this assay, serum levels < 5 mg/dL are considered as normal values, those of ⩾ 5 mL/dL are considered as indicators of existing pathology.

Statistical analysis

The SSPS 12.0 software package (SSPS, Chicago, IL, USA: 1999) and SAS 8.01 were used for statistical analysis. The patients were divided into two groups according to the C-reactive protein concentration measured preoperatively: those with CRP values in the normal range (< 5 mg/dL) and those with elevated CRP levels (⩾ 5.0 mg/dL). Median values with ranges (minimum-maximum) as well as absolute (relative) frequencies are shown for both groups. The χ^2-test with Pearson correction (χ^2) and Fisher's exact test (F) were used for the comparison of the categorical prognostic factors between the two groups. The t-test (t) was used for the comparison of continuous prognostic factors with symmetrical empirical distribution of the data. The Mann-Whitney U-test (U) served for the comparison of the other prognostic factors. The log-rank test (LR) was used for the comparison of survival times in the two groups. Morbidity, mortality, and long-term survival were assessed by multivariate analysis using Cox's proportional hazards regression model (HR). The logarithm was found for CRP, which served as a continuous variable. Backward variables selection was carried out with the Wald test (W) to the level of $\alpha = 0.05$.

Since no adjustment was made for the problem of multiple testing, all P values are descriptive.

Table 1 Tumour characteristics

Parameter	CRP < 5.0 mg/dL (n = 127)	CRP ⩾ 5.0 mg/dL (n = 164)	P-value
Histologic type (n = 289)			
-squamous cell carcinoma	56 (44%)	89 (55%)	
-adenocarcinoma	62 (49%)	60 (37%)	0.150
-undifferentiated	6 (5%)	12 (7%)	
-other	3 (2%)	1 (1%)	
Location (n = 288)			
-upper third	9 (7%)	15 (9%)	
-middle third	42 (33%)	49 (30%)	0.748
-lower third	76 (60%)	97 (60%)	
Tumour size (cm) (n = 281)	3 (0.5-12)	4.7 (0.7-20)	0.0001
Lymph nodes (n = 285)			
-dissected	23.5 (0-71)	27 (2-86)	0.015
-positive	1 (0-24)	2 (0-41)	0.015
pT-category (n = 287)			
-pT1	25 (20%)	19 (12%)	
-pT2	34 (27%)	31 (19%)	0.055
-pT3	61 (49%)	98 (60%)	
-pT4	5 (4%)	14 (9%)	
pN-category (n = 286)			
-pN0	54 (43%)	52 (32%)	
-pN1	69 (56%)	107 (66%)	0.062
-pN2	1 (1%)	3 (2%)	
pM-category (n = 283)			
-pM0	96 (78%)	116 (73%)	0.177
-pM1	27 (22%)	44 (28%)	

RESULTS

Preoperative CRP concentrations in the normal range (< 5 mg/dL) were found in 127 (43.6%) patients, elevated CRP levels were found in 164 (56.4%) patients. There were no significant differences between the two groups in age, gender, and ASA-classification ($P > 0.05$). No differences were observed for tumour type and location, although tumour extension (in cm) (measured from surgical specimen fixed in formalin) ($P < 0.0005$), the number of dissected ($P = 0.015$) and positive lymph nodes ($P = 0.015$) were significantly higher in the group with elevated CRP levels. The distribution of pT-, pN-, and pM-categories was similar in both groups ($P > 0.05$) (Table 1).

Table 2 Perioperative course

Parameter	CRP < 5.0 mg/dL (*n* = 127)	CRP ≥ 5.0 mg/dL (*n* = 164)	*P*-value
Blood transfusion (*n*) (*n* = 276)	0 (0-22)	1.5 (0-38)	0.006
ICU stay (d) (*n* = 276)	9.5 (2-107)	11 (1-115)	0.094
Internal complications (*n* = 288)	34 (27%)	74 (45%)	0.002
Surgical complications (*n* = 280)	46 (36%)	66 (41%)	0.469
-anastomotic leakage	22 (18%)	34 (21%)	0.549
-transplant necrosis	3 (3%)	7 (4%)	0.522
-mediastinitis	2 (2%)	3 (2%)	0.999
30-d-mortality (*n* = 286)	9 (7%)	12 (7%)	0.999
Hospital mortality (*n* = 280)	14 (11%)	21 (13%)	0.719

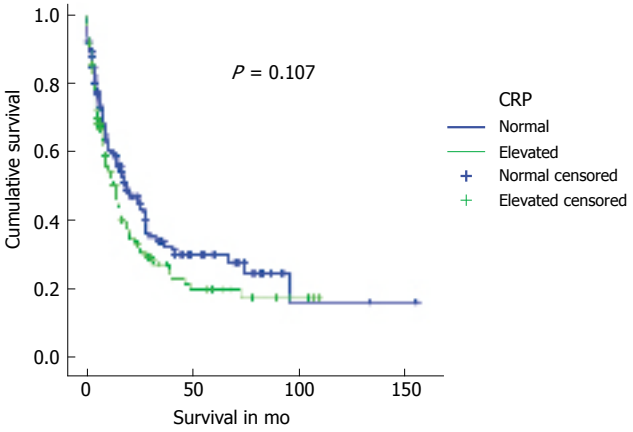


Figure 1 Kaplan-Meier survival curves for patients with elevated compared to normal preoperative CRP-values.

Perioperative course

Of the perioperative parameters, both the volume of blood transfusions ($P = 0.006$) and the number of internal medical complications ($P = 0.002$) were higher in patients with preoperative elevated CRP levels than in those with normal serum concentrations. No differences were found between the two patient groups in the length of the postoperative intensive care unit stay, the prevalence of surgical complications, or the morbidity and mortality rates (Table 2). Separate consideration of the two tumour entities showed that the squamous cell carcinoma was associated with a higher number of internal ($P = 0.010$) and surgical complications ($P = 0.083$) without a difference in the 30 d and hospital mortality rate, than adenocarcinoma where no significant differences were identified ($P > 0.05$). The comparison of the two groups showed that the preoperative CRP had no significant influence on the morbidity rate.

Long-term prognosis

A median survival of 13.6 (0-109.8) mo in patients with preoperative elevated CRP levels was markedly poorer compared to 18.9 (0-155.4) in those with normal CRP levels ($P = 0.107$). Estimated long-term survival rates calculated by the Kaplan-Meier method for both patient groups are shown in Figure 1.

For multivariate analysis, a model for the prediction of survival time was established by carrying out a backward variables selection, comprising patient age, gender, BMI (body mass index), ASA classification, computed CRP logarithms, tumour type and location, R-classification, pT-, pN- and pM-category, as well as tumour size. The data analysis was only done on those 253 patients in whom all data were available. The analysis identified that the preoperative CRP with a hazard ratio of 1.182 (95% confidence interval: 1.030-1.356) was as an independent factor, which has an influence on the long-term prognosis in patients with oesophageal carcinoma ($P = 0.0172$).

Additional relevant prognostic factors included the pT-category ($P < 0.0001$), R-classification ($P = 0.0113$), and the surgical procedures in which transthoracic was more favourable with regard to long term-prognosis than transhiatal ($P = 0.0345$) (Table 3). The forward likelihood ratio variables selection yields a similar model. A separate analysis of each of the two histologic tumour types did not identify CRP as an independent prognostic factor (squamous cell carcinoma: $P = 0.062$; adenocarcinoma: $P = 0.063$). However, multivariate analysis showed that the surgical procedure ($P = 0.0054$) was the most significant prognostic indicator on survival in patients with squamous cell carcinoma, whereas the pT-category ($P = 0.0002$) was the most significant prognostic indicator for survival in patients with adenocarcinoma.

Table 3 Independent prognostic factors in 253 patients with oesophageal resection for carcinoma

Variable	Hazard ratio	95% confidence interval (CI) Lower border	Upper border	*P*-value
pT-category[1]				< 0.0001
-pT2	2.316	1.158	4.629	0.0174
-pT3	4.240	2.211	8.131	< 0.0001
-pT4	4.432	1.854	10.596	0.0008
R-classification[2]				0.0113
0	1.992	1.231	3.224	0.0050
-R2 and R2	-0.618	0.190	2.008	0.4234
CRP (logarithm)	1.182	1.030	1.356	0.0172
Surgical approach[3]				0.0345
-transthoracic	0.680	0.486	0.953	0.0249
-transhiatal	0.326	0.079	1.338	0.1196

[1] Reference category: pT0; [2] Reference category: R0; [3] Reference category: transhiatal.

DISCUSSION

C-reactive protein as a marker of malignant potential in oesophageal carcinoma

In the present study, patients with preoperatively elevated CRP concentrations had significantly larger tumours, a higher number of lymph nodes affected by metastatic spread with a higher total number of dissected lymph nodes. The correlation between acute-phase protein

and biological behaviour in oesophageal cancer may be attributable to an inflammatory reaction, the endogenous response to tumour invasion[6,7].

The production of CRP is regulated by proinflammatory cytokines, in particular IL-6, IL-1 and TNFα, which serve as autocrine growth factors in neoplastic processes[2,8-11]. Acute-phase reactions of the organism have further been observed in tumour progression and tumour recurrence[12]. A correlation has been established between elevated serum CRP concentrations and malnutrition as well as impaired immunity in patients with oesophageal cancer[1].

While acute bacterial infections and collagen diseases are associated with a marked serum CRP elevation (> 10 mg/dL) at a latency period of 8-12 h for infections, only moderately elevated serum levels (< 10 mg/dL) have been observed in patients with malignomas[6,13].

Nozoe *et al* first presented immunohistochemical evidence of CRP expression in tumour cells of patients with squamous cell carcinoma of the oesophagus. The authors found that elevated serum levels of the protein, in addition the synthesis by hepatocytes as a response to the tumour, may be at least in part due to the production of CRP by the tumour itself[4]. In their study, the immunohistochemical expression of CRP in the tumour correlated with preoperative serum CRP levels. There were, however, no significant differences in the clinicopathologic characteristics, in particular the TNM-classification, between the patients with and without CRP expression of the tumour[4].

Comparable data on the adenocarcinoma have not been published to date.

C-reactive protein as a prognostic indicator of the perioperative course

Mortality rates after oesophagectomy cited in the literature range from 2%-25%[14]. These rates are in particular accounted by the development of pulmonary complications[15-17]. Various risk analyses have further described the impaired function of heart, liver and respiratory system, in addition to patient age and general status as independent predictors of perioperative morbidity and mortality[14,18-20]. A preoperative marker for the assessment of the intraoperative and postoperative course has not yet been established.

There was an increase in the required transfusion of packed erythrocytes in our patients with preoperatively elevated CRP, which may be due to the presence of larger tumours leading to extended operative times in this group. Furthermore, there were a greater number of general complications, especially those of pulmonary origin, in patients with a preoperative CRP value of ⩾ 5.0 mg/dL. With a view to the described cytokine interaction, Katsuta *et al* found a close association between preoperative TNF-α and IL-1β-production, and pulmonary complications after transthoracic oesophagectomy[21]. In their study, however, they did not find that either preoperatively determined CRP levels or IL-6 had an influence on the development of postoperative pneumonia[21]. A limitation of the assessment of the importance of C-reactive protein in our patient population is that the abdominothoracic procedure, which is associated with a higher pulmonary morbidity rate, was selected more frequently (80/134 patients) than the transhiatal approach (80/151 patients) in the group with elevated CRP concentrations. The difference in the occurrence of general complications was therefore of relevance only in patients with squamous cell carcinoma, but not in those with adenocarcinoma.

Significance of C-reactive protein for the long-term prognosis

In addition to the pT-category as the most significant prognostic factor, R-classification and the surgical procedure, preoperatively determined CRP was an independent prognostic factor in our patient population after surgical treatment for oesophageal carcinoma.

A number of different molecular biologic analyses have established a correlation between the expression of epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), cyclin D, int-2, hst-1, p53-mutation, CD44-variants, MIB-1-proliferation and carcinogenesis in oesophageal carcinoma and its prognosis[22-28].

The importance of CRP as a prognostic indicator in oesophageal carcinoma has recently been described in patients with squamous cell carcinoma only[3,4,29], as well as by a study consisting to 6.5% of patients with adenocarcinoma[1]. Thus far there have been no studies conducted to investigate the role of CRP in patients with adenocarcinoma, or in patients comprising with both adenocarcinoma and squamous cell carcinoma. A search of the available literature demonstrates that this study is the first to identify preoperative CRP as an independent prognostic factor for long-term survival in a mixed patient population. The number of patients with squamous cell carcinoma was only slightly higher than that with adenocarcinoma (145/291 *vs* 122/291, respectively).

In summary, the results of this study show that preoperative CRP is an easily determined and independent prognostic marker in patients with squamous cell carcinoma and adenocarcinoma of the oesophagus. The significance of the role of this marker needs to be assessed in relation to that of other important prognostic factors of long-term survival, i.e. the pT-category and R-classification. However, the pT-category and R-classification become apparent only postoperatively, the assessment of CRP is a useful tool in the evaluation of the course prior to surgical intervention.

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RAPID COMMUNICATION

N-acetylcysteine does not prevent post-endoscopic retrograde cholangiopancreatography hyperamylasemia and acute pancreatitis

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Abstract

AIM: Acute pancreatitis (AP) is the most common and often severe complication of endoscopic retrograde cholangiopancreatography (ERCP). The early step in the pathogenesis of acute pancreatitis is probably the capillary endothelial injury mediated by oxygen-derived free radicals. N-acetylcysteine - a free radical scavenger may be potentially effective in preventing post-ERCP acute pancreatitis and it is also known that N-acetylcysteine (ACC) can reduce the severity of disease in experimental model of AP.

METHODS: One hundred and six patients were randomly allocated to two groups. Fifty-five patients were given N-acetylcysteine (two 600 mg doses orally 24 and 12 h before ERCP and 600 mg was given iv, twice a day for two days after the ERCP). The control group consisted of 51 patients who were given iv. isotonic saline twice a day for two days after the ERCP. Serum and urine amylase activities were measured before ERCP and 8 and 24 h after the procedure. The primary outcome parameter was post-ERCP acute pancreatitis and the secondary outcome parameters were differences between groups in serum and urine amylase activity.

RESULTS: There were no significant differences in the rate of post-ERCP pancreatitis between two groups (10 patients overall, 4 in the ACC group and 6 in the control group). There were also no significant differences in baseline and post-ERCP serum and urine amylase activity between ACC group and control group.

CONCLUSION: N-acetylcysteine fails to demonstrate any significant preventive effect on post-ERCP pancreatitis, as well as on serum and urine amylase activity.

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Key words: N-acetylcysteine; ERCP; Acute pancreatitis; Hyperamylasemia

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INTRODUCTION

Since their introduction, endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic sphincterotomy (EST) have played a very important role in the diagnosis and treatment of biliary and pancreatic diseases. Magnetic resonance cholangiopancreatography (MRCP) is a very safe procedure and capable of providing diagnostic information similar to ERCP, but ERCP and EST still hold a key therapeutic role[1-3]. Although these procedures are generally considered being safe, they are associated with some complications, like cholangitis, haemorrhage, perforation and acute pancreatitis (AP). Acute pancreatitis is the most frequent major complication of ERCP. It is estimated that it occurs in 1%-15% of patients overall[4,5], with a higher incidence in subjects who have had therapeutic ERCP[6].

Post-ERCP pancreatitis usually is defined as new onset of abdominal pain persisting for more than 24 h after the procedure, and elevation of serum pancreatic enzymes 5 times above the normal limit. Asymptomatic increase in pancreatic enzyme activities may occur in up to 70% of all cases. Although the pathogenesis of post-ERCP pancreatitis is not clearly understood, it seems that the patient's inflammatory response to pancreatic

duct imaging and instrumentation plays a critical role[5]. An early step in the pathogenesis of acute pancreatitis is capillary endothelial injury manifested as increased capillary permeability[7]. An experimental study suggested that capillary injury might be mediated by oxygen-derived free radicals[8]. The manifestations of pancreatitis in experimental animal model can be ameliorated by blocking the action of oxygen-derived free radical scavengers[9-11]. Acetylcysteine is an excellent source of sulfhydryl groups, and is converted in the body into metabolites capable of stimulating glutathione synthesis and acting directly as free radical scavenger[12]. It has been shown that acetylcysteine can reduce severity of experimental acute pancreatitis[9,13,14]. Anecdotal reports suggest also that NAC could be effective in reducing complication of AP in humans[15]. N-acetylcysteine thus may be potentially effective in the prevention of post-ERCP acute pancreatitis.

Recently Katsinelos *et al*[16] have found that intravenous N-acetylcysteine does not prevent post-ERCP pancreatitis. The authors have pointed out that their findings cannot be generalized because larger cohorts may be needed to definitevly elucidate this problem. As combination of data from multiple, small (even underpowered) trials should enhance the precision and accuracy of any meta-analysis[17], we describe here the results of a prospective, randomized open-label study to determine the effect of N-acetylcysteine on the incidence of hyperamylasemia and pancreatitis after ERCP, which was previously reported only in the abstract form[18].

MATERIALS AND METHODS

Patients

Between January and December 2002, 345 consecutive patients undergoing ERCP in our institution were assessed for eligibility to enter the study. A total of 106 patients who fulfilled the entry criteria and gave their consent, were enrolled into the study. Exclusion criteria were as follows: presence of acute pancreatitis and/or severe chronic pancreatitis and/or hyperamylasemia, pregnancy and breast feeding, age under 18.

Patients were randomized to one of the two groups. In the treated group, 55 subjects received N-acetylcysteine (*ACC 600*, Hexal-Polska). Two 600 mg doses were given orally 24 h and 12 h before ERCP and 600 mg was given intravenously, twice a day for two days after the ERCP. The control group consisted of 51 patients who were given iv isotonic saline twice a day for two days after the ERCP. The present study was approved by the local ethics committee.

Endoscopic procedure

All endoscopic procedures were performed by the same experienced endoscopist. As a contrast medium 60% dilution of diatrizoate meglumine was used. All patients were fasted overnight and remained fasting for a minimum of 12 h after the ERCP. Patients stayed in the hospital for at least 48 h after the procedure. Biochemical parameters and clinical status were examined. Premedication consisted of pharyngeal anesthesia with xylocaine spray and intravenous administration of midazolam and hyoscine-N-butyl bromide titrated according to age and tolerance. ERCP was performed with the patient under general anesthesia with propofol and fentanyl, without airway intubation. At the end of each procedure, the endoscopist recorded also the details of the maneuvers performed, especially the ease or difficulty of cannulation, number of cannulations, number of pancreatic duct injections, presence of parenchymogram on radiography, or whether a needle-knife sphincterotomy was performed. Biochemical and clinical evaluation, serum and urine amylase activity were measured before ERCP and after 8 and 24 h of the procedure. Before the endoscopic procedure and 24 h after, blood samples were analysed for alanine and asparagine transaminases, bilirubin and white blood cells (leukocytosis was defined as a white cell count greater than 10000/ mm^3). The primary outcome parameter was frequency of AP post-ERCP, defined by clinical features consistent with acute pancreatitis beginning after ERCP and lasting for at least 24 h, associated with increase in serum amylase levels greater than five times above normal.

Secondary outcome parameters were differences between groups in serum and urine amylase activities measured at 8 and 24 h after the ERCP.

Table 1 Indications for ERCP in N-acetylcysteine treated group (ACC-group) and control group

ERCP indications	ACC group	Control group	*P*
Bile duct stones	35	32	NS
Recurrent pancreatitis	2	4	NS
Biliary pain, cholestasis	3	5	NS
Chronic pancreatitis	8	3	NS
Pancreatic cancer	3	1	NS
Other	4	6	NS

Statistical analysis

Univariate statistical analysis was performed using chi-square test, Fisher's exact test for count data or Student's *t*-test for comparison of means. Multivariate analysis was performed using forward logistic regression procedure and 0.05 and 0.1 as values to enter or remove the variables. Serum and urine amylase data were subjected to repeated measure analysis of variance with N-acetylcysteine treatment as between subject factor. $P < 0.05$ was considered statistically significant. The STATA software, version 8.2 for Macintosh (Stata Corporation, College Station, TX, USA) was used for statistical computations.

RESULTS

Of the 106 patients included, 55 were randomized to the ACC group and 51 to the control group. The groups were similar with respect to the main indications for ERCP (Table 1) and endoscopic procedures performed (Table 2). There were no significant differences in acute pancreatitis risk factors (female gender, multiple cannulations, pancreatic duct injections, precut papillotomy, common biliary duct diameter; diagnostic ERCP and failed procedure) between the groups (Table 3). Post-ERCP pancreatitis graded as mild or moderate was observed in 10 (9.4%) patients, 4

Table 2 ERCP procedure performed

ERCP procedure (patients)	ACC group	Control group
Diagnostic	4	2
Therapeutic	51	49
Endoscopic sphincterotomy	44	47

Table 3 Risk factors for acute pancreatitis

Risk factors (patients)	ACC group	Control group	Total	*P*
Gender (F/M)	33/22	33/18	66/40	NS
Multiple cannulations	9	7	16	NS
Pancreatic duct injections	13	12	25	NS
Diagnostic ERCP	5	1	6	NS
Failed procedure	2	3	5	NS
Precut papillotomy	12	9	21	NS
Small bile duct diameter	2	5	7	NS

Table 4 Unvariate analysis of group differences according to the presence of acute pancreatitis

Variables	Acute pancreatitis (*n* = 10)	No acute pancreatitis (*n* = 96)	*P*
N-acetylcysteine / Control	4/6	51/54	NS
Sex (F/M)	9/1	57/39	NS
Precut (Y/N)	6/4	15/81	0.004
Small bile duct diameter (Y/N)	1/9	6/90	NS
Multiple cannulations (Y/N)	5/5	11/85	0.006
Pancreatic duct injections (Y/N)	4/6	21/75	NS
Diagnostic ERCP (Y/N)	1/9	5/91	NS

(7.3%) patients in the ACC group and 6 (11.8%) in the control group (NS). In univariate analysis of differences between two groups according to the presence of acute pancreatitis, only two factors were significantly associated with an increased risk of pancreatitis: precut papillotomy and multiple cannulations. Female sex was on the verge of statistical significance (Table 4).

There were no significant differences in baseline and post-ERCP serum and urine amylase activity between ACC group and control group (Table 5). There were also no differences in total bilirubin, alanine and aspartate aminotransferases and WBC between the two groups (data not shown).

Initially the following variables including N-acetylcysteine treatment, precut papillotomy, multiple cannulations, common biliary duct diameter, injection of the contrast into pancreatic duct, failed procedure, sex and age, were entered into a logistic regression model.

On multiple logistic regression analysis, only precut papillotomy remained as a predictor of acute pancreatitis (Table 6).

DISCUSSION

Although certain risk factors have been identified, the development of post-ERCP pancreatitis remains unpredictable. The knowledge of risk factors for post-ERCP pancreatitis is often not helpful in avoiding pancreatitis in individual patients. For these reasons it would be beneficial to find prophylactic treatment capable of preventing post-ERCP pancreatitis. The present study was conducted to test the hypothesis that N-acetylcysteine might reduce the frequency and intensity of post- ERCP pancreatitis. Our results did not support this hypothesis. Similarly many other therapeutic agents including octreotide, low-molecular weight heparin, nifedipine, have no beneficial effect on the frequency of post-ERCP pancreatitis[19-26]. On the other hand, there are data suggesting that gabexate mesylate, diclofenac and heparin may reduce the frequency of post-ERCP pancreatitis[5,27-30]. Data concerning corticosteroids, IL-10 and somatostatin are contradictory[19, 31-33].

Oxygen reactive species (ROS) have been implicated in the initiation of acute pancreatitis[34]. Animal models of acute pancreatitis point out to the occurence of early and profound oxidative stress[35,36]. In human studies of acute pancreatitis, increased blood concentrations of the superoxide radical and lipid peroxides, as well as depletion of ascorbic acid and an increased fraction of dehydroascorbic acid have been found[37].

In experimental pancreatitis various forms of scavenger therapy for ROS, have been shown to mitigate the pancreatic tissue damage after induction of acute pancreatitis and to attenuate the extrapancreatic complications, suggesting that superoxide dismutase[35], polyethylene glycol-linked superoxide dismutase[38], allopurinol[11], CV 3611 (a synthetic ascorbic acid derivative)[10] and N-acetylcysteine[9,13], may play their role in attenuating the extrapancreatic complications. N-acetylcysteine is converted to metabolites capable of stimulating glutathione synthesis[12] and is an important antioxidant and essential cofactor for antioxi-

Table 5 Serum and urine amylase activity before ERCP, after 8 and 24 h in control and ACC groups (mean ± SD)

Serum and urine amylase activity levels		Control group (%)	ACC group (%)	*P*
before ERCP	serum	62.1 ± 59.7	79.0 ± 79.4	NS
	urine	272.9 ± 320.5	268.1 ± 355.2	NS
8 h after ERCP	serum	324.6 ± 487.1	270.1 ± 484.7	NS
	urine	596.3 ± 1108.3	775.9 ± 1500.4	NS
24 h after ERCP	serum	268.6 ± 590.9	183.56 ± 252.1	NS
	urine	1324.8 ± 2763.9	1033.7 ± 2499.6	NS

Table 6 Summary of the results of forward selection multiple logistic-regression analysis with development of post-ERCP acute pancreatitis as the outcome variable

Variable[1]	Odds ratio	95% CI	*P*
Precut (no precut *vs* precut)	0.108	0.026-0.456	0.003
Sex (F *vs* M)	7.432	0.841-65.689	0.071

[1] Other variables that were examined but did not enter the model including N-acetylcysteine treatment, multiple cannulations, bile duct diameter < 8 mm, injection of contrast into pancreatic duct, failed procedure and age.

dant enzymes, and is depleted in AP[39].

Nuclear factor-kappaB (NF-κB), an oxidant-sensitive transcription factor, may regulate the induction of cytokine gene expression, which in turn regulates inflammatory response[13]. NF-κB activation and cytokine production in pancreatic acinar cells are suppressed by N-acetylcysteine[13,40]. It was reported that of ACC use in patients suffering from acute pancreatitis and multiorgan failure can improve their clinical status[15].

The occurrence of pancreatic damage after ERCP may be due to extraductal leakage of the pancreatic juice present in the ducts, rupture of acini or alterations of the acinar cells. Such functional alterations cause leak of pancreatic enzymes into the interstitium and then into the blood, consequently leading to hyperamylasemia and hyperamylasuria. In our study, ACC treatment had no influence on post-ERCP serum and urine amylase activities, suggesting that ACC does not ameliorate pathophysiologic mechanisms operating post-ERCP, leading to leak of pancreatic enzymes.

Our results are consistent with those obtained by Katsinelos *et al*[16]. There are however some differences between the present study and that of Katsinelos and colleagues[16]. We used a lower dose of N-acetylcysteine, which is similar to that used for the prevention of nephrotoxicity by contrast agents[41]. Although in AP experimetal models doses are used as high as 1000 mg/kg[9], there is evidence that high doses might actually have deleterious effects on the course of AP by inducing apoptosis probably due to inhibiting NF-κB[42,43].

Although our study was underpowered, it should be noted that increasing sample size would probably not yield different results. We initially assumed 10% rate of post-ERCP pancreatitis, it would be 3% in the treatment group. Then the number of patients required for the study with a two-sided 5% significance test and a power of 80% was 194 per group. Interim analysis was performed after inclusion of 106 patients to determine if a larger sample might change findings. An effect size of 0.077 calculated from the power analysis indicated that a statistically significant difference did not occur even if the study was continued and enrolled preplanned number of patients. In fact it would be necessary to recruit at least 669 subjects in each group to demonstrate any statistical significance, a clearly untenable goal. In addition, N-acetylcysteine treatment had no statistically significant influence on serum amylase activity. Therefore a decision to terminate the study was made.

We also attempted to identify conditions predisposing patients to the development of AP post-ERCP. In the present study, precut papillotomy was significantly associated with increased risk of pancreatitis, suggesting that this factor reflects the technical difficulty of the procedure.

In conclusion, N-acetylcysteine fails to demonstrate any significant preventive effect on post-ERCP pancreatitis, as well as on serum and urine amylase activity. Pancreatic injury is related to endoscopic maneuvers performed to obtain biliary access, rather than to the patient-related factors. Hence, a therapeutic agent with significant prophylactic effect on post-ERCP pancreatitis is still needed and it is necessary to perform more studies[44].

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RAPID COMMUNICATION

Risk factors for retinopathy associated with interferon α-2b and ribavirin combination therapy in patients with chronic hepatitis C

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Abstract

AIM: To elucidate the frequency and risk factors for retinopathy in patients with chronic hepatitis C who are treated by interferon-ribavirin combination therapy.

METHODS: We prospectively analyzed 73 patients with histologically confirmed chronic hepatitis C, who underwent combination therapy for 24 wk. Optic fundi were examined before, and 2, 4, 12 and 24 wk after the start of combination therapy.

RESULTS: Fourteen patients (19%) developed retinopathy, which was initially diagnosed by the appearance of a cotton wool spot in 12 patients. Retinal hemorrhage was observed in 5 patients. No patient complained of visual disturbance. Retinopathy disappeared in 9 patients (64%) despite the continuation of combination therapy. However, retinopathy persisted in 5 patients with retinal hemorrhage. A comparison of the clinical background between the groups with and without retinopathy showed no significant differences in age, gender, viral genotype, RNA level, white blood cell count, platelet count, prothrombin time, complications by diabetes mellitus or hypertension, or pretreatment arteriosclerotic changes in the optic fundi. However, multiple logistic regression analysis revealed that complication by hypertension was observed with a high frequency in the group with retinopathy (P = 0.004, OR = 245.918, 95% CI = 5.6-10786.2).

CONCLUSION: Retinopathy associated with combination therapy of interferon α-2b and ribavirin tends to develop in patients with hypertension.

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Key words: Retinopathy; Ribavirin; Chronic hepatitis C; Interferon

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INTRODUCTION

Chronic hepatitis C, which affects more than 170 million people in the world[1], may eventually lead to cirrhosis and/or hepatocellular carcinoma. The main treatment for this intractable disease is interferon administration. Published guidelines recommend interferon-ribavirin combination therapy as a first-line treatment[2]. Interferon is also used in the treatment of other viral and neoplastic diseases.

Various adverse effects have been reported due to use of interferon[3]. An influenza-like syndrome, characterized by fever, chills, myalgias, arthralgias, and headache, is the most common adverse effect. Toxicities of the central nervous, hematopoietic, gastrointestinal, urinary, cardiovascular, musculoskeletal and endocrine systems have also been described. However, ocular toxicity was not reported before the use of interferon for chronic hepatitis[3].

After the introduction of interferon for the treatment of hepatitis, retinal complications have been reported. Hayakawa *et al* showed that 17 of 43 patients developed retinopathy during interferon monotherapy. They also showed that the prevalence of retinopathy was higher in patients with diabetes[4]. Subsequently, several papers have shown that a substantial proportion of patients undergoing interferon monotherapy develop retinopathy[5-7]. However, the prevalence of retinopathy is variable, which is

presumably attributed to the difference in the treatment regimen and/or background of patients.

As mentioned above, interferon-ribavirin combination therapy has become the standard treatment for chronic hepatitis C. Results from recent studies have suggested that the prevalence of retinopathy associated with combination therapy may be higher than that associated with interferon monotherapy, which should be further investigated[8-10].

In spite of the high prevalence, risk factors for interferon-associated retinopathy are still unclear. Diabetes mellitus and the patients' age were reported to be possible risk factors for retinopathy associated with interferon monotherapy[4]. In interferon-ribavirin combination therapy, diabetes, hypertension[8], and response to treatment[10] were considered possible risk factors. However, the results are not conclusive because of the small number of patients examined.

The aim of the present study is to elucidate the prevalence and risk factors for retinopathy associated with interferon-ribavirin combination therapy.

MATERIALS AND METHODS

Patients

Seventy-three consecutive patients with histologically confirmed chronic hepatit is C (47 males and 26 females; median age, 53.4 years; ranges 26-73 years) were enrolled in this study from 2002 to 2004. The clinical backgrounds of the enrolled patients are shown in Table 1. All patients were treated with recombinant interferon α-2b (Intron A, Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ, USA) combination therapy. All the patients were treated daily with interferon α-2b at 6 MU for 2 wk followed by three times a wk treatment with interferon α-2b at 6 MU for 22 wk in combination with ribavirin. Ribavirin was given orally twice a day at a total daily dose of 600 mg for patients who weighed 60 kg or less and 800 mg for the remaining patients who weighed more than 60 kg for 24 wk.

All patients were assessed to determine the safety, tolerance, and efficacy of the treatment at the end of wk 1, 2, 4, and every 4 wk during the treatment. After the treatment was completed, patients were followed up on wk 4, 8, 12, and 24. The primary end point was indicated by a sustained loss of detectable HCV-RNA at 24 wk after the treatment.

Methods

Optic fundi were examined before, and 2, 4, 12 and 24 wk after the start of combination therapy. Ophthalmological examinations were carried out before the start of treatment and 2, 4, 12 and 24 wk after the start of treatment until the completion of treatment or until the retinopathy disappeared. Fundus photographs were taken for documentation and comparison when retinal abnormalities were detected.

Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committees of our institutions.

Table 1 Profiles and initial laboratory data of patients with and without retinopathy during IFN-ribavirin combination therapy[1]

	Total	Retinopathy (+)	Retinopathy (-)
Patients			
Number	73	14	59
Age (yr)	53.4 ± 10.9	56.3 ± 10.5	52.8±38.6
Gender (M/F)	47/26	10/4	37/22
Hypertension (Yes/No)[a]	15/58	5/8	10/49
Diabetes mellitus (Yes/No)	2/71	1/13	1/58
Peripheral blood count			
Platelet count ($\times 10^4/mm^3$)	15.3 ± 6.0	12.5 ± 10.5	15.9 ± 38.6
White blood cell ($\times 10^3/mm^3$)	46.9 ± 12.6	46.5 ± 13.0	48.6 ± 10.9
Hemoglobin (×g/dL)	14.0 ± 1.3	14.0 ± 1.0	14.0 ± 1.4
Prothrombin time (%)	90.2 ± 13.3	87.1 ± 13.3	90.8 ± 13.3
ALT (IU/L)	109.4 ± 78.2	104.1 ± 41.0	110.4 ± 83.6
Viral factors			
Genotype (type 1/type 2)[2]	45/26	33/24	12/2
Viral load (kcopies/mL)	592.3 ± 271.2	505.6 ± 309.1	607.5 ± 271.2
Pretreatment/ Arteriosclerotic	12/61	7/7	5/54
changes in optic fundi (Yes/No)			
Response to therapy (SVR/non-SVR)	38/35	5/9	33/26

[1] Data are expressed as mean ± SD.
[2] Genotype could not be determined in 2 patients.
[a] $P = 0.004$

RESULTS

Before the start of the combinaiton therapy, one patient had scars from laser coagulation of a previous interferon-associated retinopathy and another patient had retinal central vein occlusion. Arteriosclerotic changes of the optic fundi were observed in 12 patients.

After the start of interferon-ribavirin combination therapy, 14 out of 73 patients (19%) developed retinopathy. The clinical profiles and laboratory data of the patients with and without retinopathy are shown in Table 1.

We compared the characteristics of patients who developed retinopathy and those who did not. The two groups showed no statistical differences in age, gender, subtype of virus, RNA level, white blood cell count, platelet count, prothrombin time before treatment or prevalence of pretreatment fundic arteriosclerotic changes. The patients with retinopathy were more frequently complicated by hypertension ($P = 0.004$) (Table 1).

Logistic regression analysis of factors affecting retinopathy was also carried out. Hypertension was found to be a factor for predicting retinopathy (Table 2).

Table 3 shows the optic fundi findings of the 14 patients with retinopathy. Retinopathy was initially diagnosed by the appearance of a cotton wool spot in 12 patients. In three of the 12 patients, retinal hemorrhage was also observed simultaneously or sequentially. Two of the 14 patients who developed retinopathy were diagnosed by retinal hemorrhage without a cotton wool spot. No patient complained of the visual disturbance.

Table 2 Logistic regression analysis of factors associated with retinopathy

Factor	*P*	Odds ratio	95% confidennce interval
Sex	0.68	1.699	0.1-21.0
Age	0.203	1.099	1.0-1.3
Genotype	0.776	1.621	0.1-45.5
Levels of HCV RNA	0.114	1.006	0.99-1.0
Hypertension	0.004	246.32	5.5-10977.8
Diabetes mellitus	0.211	0.122	0.1-3.3
Abnormal findings in pretreatment optic fundi	0.904	1.192	0.1-20.3
Platelet	0.059	1.391	1.0-1.9
Prothrombin time	0.747	0.982	0.9-1.1
ALT	0.992	1	0.98-1.0
WBC	0.964	1.027	0.4-2.9
Response to therapy (SVR or non-SVR)	0.123	0.016	0.0-3.1

Table 3 Optic fundi findings of patients with retinopathy

No	Age	Sex	Underlying disease		Optic fundi before treatment		Optic fundi after treatment	
			Hyper tension	Diabetes mellitus	H	S	Cotton wool spot	Retinal hemorrhage
1	38	M	+	+	0	0	4 wk-	4 wk-
2	52	M	+	−	1	0	4-12 wk	−
3	40	M	−	−	0	0	6-36 wk	−
4	62	F	−	−	0	0	4-36 wk	−
5	61	M	+	−	0	0	12 wk-	−
6	58	M	−	−	1	1	12 wk-	−
7	73	M	−	−	2	2	4-28 wk	−
8	65	F	+	−	0	0	24-36 wk	−
9	59	F	+	−	2	2	2 wk-	4-24 wk
10	40	M	−	−	0	0	4-20 wk	−
11	62	F	−	−	1	2	2 wk-	4 wk-
12	65	M	−	−	1	1	2-24 wk	−
13	40	M	−	−	0	0	−	8-16 wk
14	40	M	−	−	0	0	−	2-4 wk

Retinopathy disappeared in 9 of the 14 patients despite the continuation of combination therapy. However, it continued in three patients with retinal hemorrhage and two without retinal hemorrhage.

Ocular manifestations other than retinopathy (e.g., ocular pain, a mild watery eye and conjunctivitis) were not observed in any patients.

DISCUSSION

Interferon associated retinopathy was first recognized in 1990 when Ikebe and associates reported a 39-year-old patient who developed retinal hemorrhages and cotton wool spots following intravenous administration of interferon[11].

The exact mechanism of interferon-induced-retinopathy is not known but is presumably related to the disturbance in retinal microcirculation[12]. Therefore, preexisting arteriosclerosis that affects microcirculation may promote interferon-induced retinopathy.

Our study shows that hypertension is a more frequent complication in patients with interferon-induced-retinopathy. Chronic hypertension is associated with the thickening of the walls of the arteries and small arterioles[13]. Therefore, systemic hypertension predisposes patients to interferon-induced-retinopathy. The fact that hypertensive retinopathy induces the formation of flame-shaped hemorrhages and white cotton wool spots, which are also seen in interferon-induced-retinopathy, implies that systemic hypertension and interferon-induced-retinopathy may be related each other.

Statistical analysis did not indicate pretreatment optic fundic changes or diabetes as predictive factors of retinopathy. This may be attributed to the following reasons: (1) pretreatment changes in the optic fundi as a predictive factor are included in hypertension; and (2) the number of patients with diabetes is too small. Regardless of these reasons, systemic hypertension is an important risk factor for interferon-related retinopathy.

The frequencies of interferon-induced retinopathy associated with interferon monotherapy and interferon-ribavirin combination therapy are reported to be 24%-58%[4-7,14,15] and 16%-64%[8-10,16], respectively. The frequency in the present study (20%) was lower than that in previous reports. Furthermore, the ocular side effects of ribavirin, which include a mild watery eye and conjunctivitis, were not seen in this study. Therefore, the frequency of induced retinopathy associated with combination therapy may be considered as high as that associated with interferon monotherapy.

Retinopathy developed by 12 wk in most (13/14, 93%) of the patients after the start of combination therapy and disappeared in majority (10/14, 71%) of the patients during the 4-8 wk period, in which the patients were receiving the treatment. This suggests that treatment can be continued despite the development of retinopathy in many patients. However, two patients who developed cotton wool spots early in the therapy (2 wk) therafter suffered from retinal hemorrhage in a prolonged manner. Therefore, patients who develop cotton wool spots early in the therapy should be carefully monitored. However, as reported in previous studies[4,8,17], most of the patients with retinopathy in this study were asymptomatic. Therefore, combination therapy may be continued in most patients.

The fact that retinopathy occurred more frequently in patients with hypertension, suggests that these patients should be carefully monitored. With periodic examination of the optic fundi, major bleeding that causes visual symptoms may be prevented or detected at an early stage. Therefore, patients who undergo interferon-ribavirin combination therapy, particularly those with hypertension, should undergo periodic examination of the optic fundi.

To conclude, retinopathy associated with combination therapy of interferon α-2b and ribavirin tends to develop in patients with hypertension.

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RAPID COMMUNICATION

Treatment of patients with advanced gastrointestinal stromal tumor of small bowel: Implications of imatinib mesylate

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Abstract

AIM: To examine the impact of imatinib mesylate (Glivec) on patient survival and response and its safety, and the correlation of the response rate with the kit gene mutation status.

METHODS: Thirty-three of 74 (44.6%) small bowel gastrointestinal stromal tumor (GIST) patients who developed recurrence after curative resection and not treated with Glivec were classified as group A patients. Twenty-two advanced small bowel GIST patients treated with Glivec were classified as group B patients. Clinicopathological features, post-recurrence and overall survival rates were compared. Each tumor in group B patients was investigated for mutations of kit or platelet-derived growth factor alpha (PDGFRA). The mutation type was correlated with clinical outcomes. The anti-tumor effect and safety of Glivec in group B patients were also assessed.

RESULTS: Advanced small bowel GIST patients treated with Glivec had substatntially longer post-recurrence survival and higher overall survival rates than those not treated with Glivec. A total of 15 patients had a partial response (PR) (67.8%). Activated mutations of c-kit were found in 16 of 19 tested patients and no PDGFRA mutant was identified. In 13 patients with GISTs harboring exon 11 kit mutations, the partial response rate (PR) was 69.3%, whereas two of three patients with tumors containing an exon 9 kit mutation had an overall response rate (ORR) of 66.7% (not significant).

CONCLUSION: Glivec significantly prolongs the post-recurrence and overall survival of Asian patients with advanced GISTs. Glivec induces a sustained objective response in more than half of Asian patients with advanced small bowel GISTs. Activated mutations of kit exon 11 are detectable in the vast majority of GISTs. There is no difference in the PR rate for patients whose GISTs have kit exon 9 and exon 11 mutations.

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Key words: Gastrointestinal stromal tumor; Glivec; Patient survival; Kit gene mutation

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are soft-tissue sarcomas primarily arising from mesenchymal tissue in the gastrointestinal (GI) tract and abdomen. They are rare neoplasms, accounting for 0.1%-3% of all gastrointestinal tract tumors[1]. However, GISTs are the most common mesenchymal malignancy of the GI tract with the precise incidence unknown[2]. Approximately 5000 GISTs are diagnosed annually in the USA, distributing equally in men and women. GISTs appear to be related the interstitial cells of Cajal of the mesenteric plexus[3]. These cells are considered as GI pacemaker cells, from the interface between the automatic innervation of the bowel wall and its smooth muscle[4,5]. GISTs express the cell-surface transmembrane receptor kit with a tyrosin kinase activity and are the protein product of the kit proto-oncogene. There are frequent gain-of-function mutations of kit in GISTs. These mutations result in constitutive activation of kit signaling, which leads to uncontrolled cell proliferation and resistance to apoptosis. It has been recently reported that kit activation occurs in all cases of GISTs, regardless of the mutation status of kit.

Surgical resection remains the mainstay of therapy for GIST. However, recurrence is common, the 5-year survival rate after complete resection ranges from 40% to 65%[6-10]. Unresectable or metastatic GIST is a fatal disease that resists conventional chemotherapy. A recently study reported that the response rate to doxorubicin therapy is less than 5%[11]. The effectiveness of radiation therapy for unresectable or metastatic GIST has not been proved.

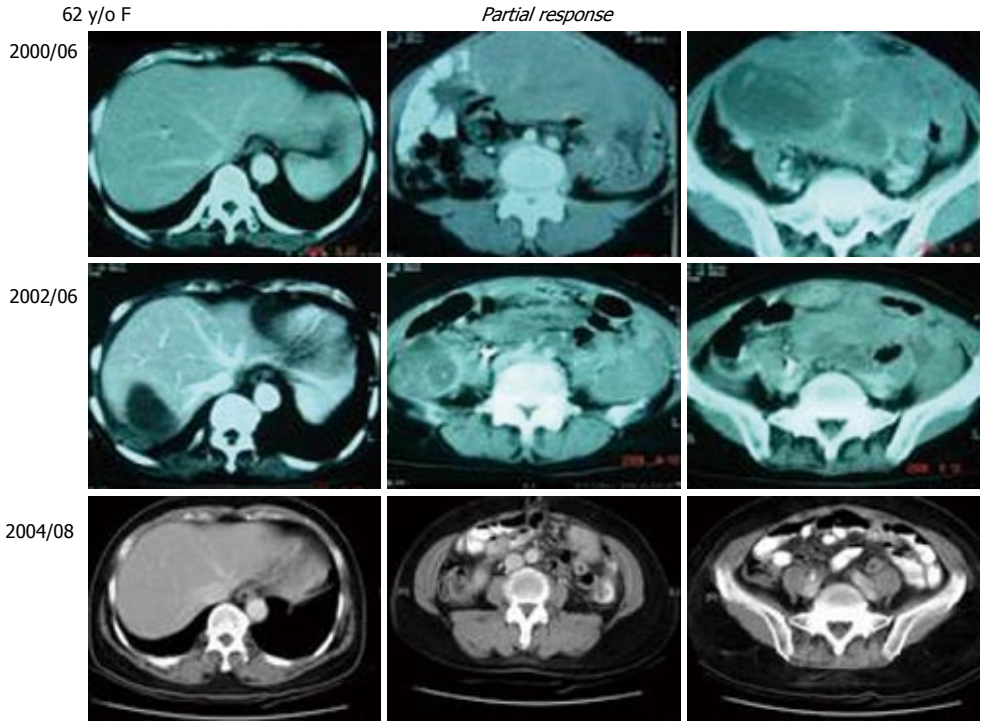


Figure 1 Sequential CT scans obtained in the same patient at base line (before treatment, upper row), middle row (2 year after Glivec treatment), and lower row (50 mo after Blivec treatment). The CT scans revealed partial response of the tumor at the same level.

The median length of survival is approximately 20 mo for patients with a metastatic GIST and 9 to 12 mo for patients with local recurrence[3]. Before the development of Glivec, the outlook for patients with advanced GIST is extremely poor. A significantly large number of patients with initial resection of GIST eventually experience recurrence, for which there is no effective treatment.

Imatinib mesylate (formerly STI571, now referred to as Gleevec or Glivec) selectively inhibits certain protein tyrosin kinases, such as intracellular ABL kinase, chimeric BCR-ABL fusion oncoprotein of chronic myeloid leukemia, transmembrane receptor kit, and platelet-derived growth factor (PDRGF) receptors[12-15]. Glivec induces a sustained objective response in more than half of patients in the West with advanced GISTs[16]. However, the implication of Glivec on patient survival, patient response to and safety of Glivec for Asian patients with advanced GIST has not been elucidated. This study examined the impact of Glivec on patient survival and response and its safety, and the correlation of the response rate with the kit gene mutation status.

MATERIALS AND METHODS

Patients

From January 1983 to December 2002, 77 small bowel GIST patients with positive kit immunostaining underwent curative resection at the Department of Surgery, Chang Gung Memorial Hospital, Taiwan. Patients with other concurrent malignancies at presentation as well as those with incidental findings of tumors on laparotomy (most tumors were < 2 cm and had trace mitotic count) were excluded. Clinical presentation, operative findings, and long-term outcomes were retrospectively reviewed. Curative operation was defined as negative resection margin observed during histopathological examination. Recurrent disease was defined as the presence of a histologically or radiographically identified tumor. Distant metastatic disease was defined as that occurring at structures noncontiguous with the primary tumor site. Regional intraperitoneal disease was deemed a local recurrence if it involved a recurrent solitary tumor or sarcomatosis.

During 2002 to June 2004, 22 histologically confirmed unresectable or metastatic small bowel GIST patients who expressed CD117 and CD34 (a marker of kit-receptor tyrosine kinase) were enrolled in this study. Criteria for inclusion were as follows: at least one measurable tumor; adequate hepatic, renal, and cardiac function; an adequate platelet count; and an Eastern Cooperative Oncology Group (ECOG) performance status of 3 or less. Patients could have previously received chemotherapeutic regimens (the last chemotherapy treatment must have been at least four weeks before study entry) and undergone radiotherapy, or surgery, or both. The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital and written informed consent for drug administration and the analysis of tumor-associated genetic alteration was obtained from each patient.

Study design

A prospective, non-randomized, and single center trial was conducted to evaluate the role of Glivec in inducing objective response in GIST patients. Patients were administered 400 mg of Glivec in 100 mg capsules, taken orally once daily with food. Patients had regular physical examinations and evaluations of performance status, body weight, complete blood count, and serum chemistry. The administration of each dose and any adverse events were recorded for each patient. Standard computed tomography (CT) was performed on each patient every three months to assess patient response (Figures 1A-C). Standard [18F]

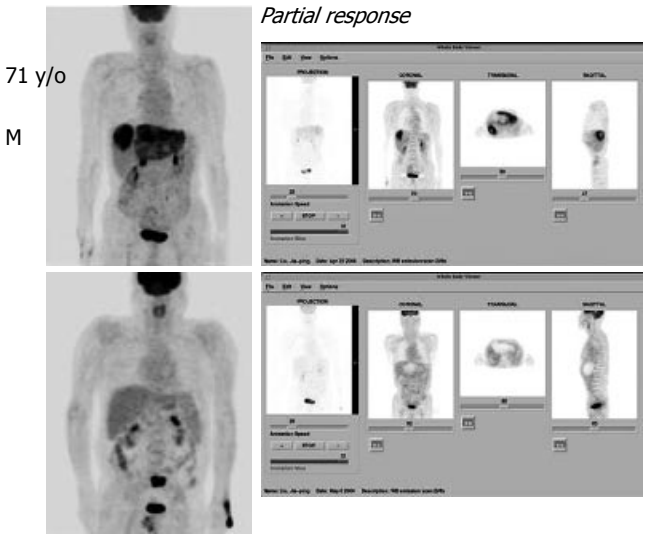


Figure 2 Sequential PET scans obtained in the same patient at base line (before treatment, upper row), and 1 mo after Glivec treatment (lower row). The PET scans revealed partial response of the tumor at the same level.

fluoro-2-deoxy-D-glucose positron-emission tomography (PET) scanning was performed on three patients to complement standard computed tomography (CT) and to assess changes in the metabolic profiles of the tumors (Figure 2).

Efficacy and safety evaluation

The response of the tumor to Glivec was evaluated after one month, three months, and every three months thereafter or whenever medical need was indicated. Assessments were performed according to the standard Southwest Oncology Group criteria and based solely on CT or PET[17]. Responses were classified as follows: complete response (CR) (disappearance of all the disease that could be measured and evaluated), partial response (PR) (> 50% of the decrease in the sum of the products of the perpendicular diameters of all measurable lesions, the absence of progression, and the absence of new lesions), stationary disease (SD) (a response that did not qualify as a complete response, a partial response, or disease progression), and disease progression (DP) (> 50 per cent increase or an increase of 10 cm, whichever was smaller) in the sum of the products of the perpendicular diameter of all measurable lesions, worsening of a lesion that could be evaluated, the reappearance of any lesion or the presence of a new lesion, or failure of the patients to return for evaluation because of disease progression. Toxic effects were recorded in accordance with the National Cancer Institute Common Toxicity Criteria[18].

Analysis of kit and PDGFRA mutations

Sections were prepared from formalin-fixed, paraffin-embedded pretreatment specimens trimmed to enrich tumor cells. Polymerase chain reaction amplification of genomic DNA for kit and PDGFRA was performed and amplification was analyzed for mutations as previously described[19] (Figure 3).

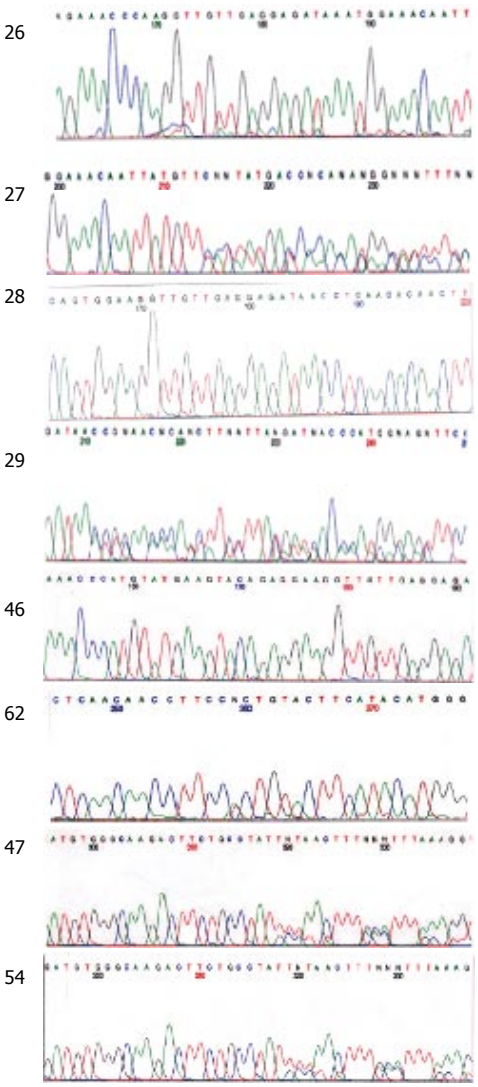


Figure 3 Mutation spectrum of the 8 small bowel GIST patients.

26, 27: Deletion mutation in exon 11; 28, 29: Deletion and insertion mutation in exon 11; 46, 62: Missense mutation in exon 11; 47, 54: Insertion mutation in exon 9.

Statistical analysis

All data were presented as percentages of patients or means with standard deviation. Numerical data were compared by an independent two-sample *t* test. Pearson chi-square test and Fisher's exact test were used for nominal variables. Survival rate was calculated and plots were constructed by the Kaplan-Meier method and compared between groups with a log-rank test. All statistical analyses were performed using SPSS computer software package (Version 10.0, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Clinical features

Table 1 presents a summary of clinical features. A similar age distribution and gender ratio were observed in the two groups. The jejunum was the most common site for small bowel GISTs treated with or without Glivec. Liver was the most frequent site for metastasis of small bowel GISTs. There was no significant difference in tumor diameter between the two groups.

Table 1 Demographic data of 55 advanced and metastatic small bowel GIST patients treated with or without imatinib meslyate

	Non-Glivec (%) (n = 33)	Imatinib meslyate (%) (n = 22)	P
Age (yr)	56.5 ± 11.1	55.7 ± 15.4	0.803
Gender (M : F)	17 : 16	13 : 09	0.58
Location			
Duodenum	4 (12.1)	5 (22.7)	
Jejunum	22 (66.7)	8 (36.4)	
Ileum	7 (21.2)	6 (27.3)	
Multiple	0 (0)	3 (13.6)	0.05
Tumor size			
Mean/Median (cm)	10.3 ± 4.9/9.0	11.7 ± 5.1/10.5	0.315
Range (cm)	3.5-25	4-24	
Recurrence			0.022
Liver	28 (84.8)	11 (50)	
Peritoneum	10 (30.3)	10 (45.5)	
Retroperitoneum	4 (12.1)	5 (22.7)	
Local	4 (12.1)	10 (45.5)	
Others	1 (0.3)	4 (18.2)	

M: Male; F: Female.

Table 2 Prognosis of 55 advanced and metastatic small bowel GIST patients treated with or without imatinib meslyate

	Non-Glivec (%) (n = 33)			Imatinib meslyate (%) (n = 22)			P
	Re-resection (n = 6)	C/T (n = 5)	Conservative treatment (n = 22)	CR (n = 0)	PR (n = 15)	SD (n = 4)	PD (n = 3)	
PRS (m) (median/mean)		6.7/14.7			-/72.6			0.00001
Mean	36.2	4.8	9.6	75.6	31.6			
Median	15.7	3.7	5.9	-	24.7			
95% CI of mean	7.5-65.0	2.3-7.3	6.1-13.0	67.5-87.0	22.0-41.2			
Log-rank (p)		0.048			0.462			
OS (m) (median/mean)		33.6/49.2			137.7/128.9			0.00001
Mean	73.1	28.5	48.2	140.1	53.2			
Median	47.5	23.5	33.2	137.7	50.9			
95% CI of mean	27.0-119.3	18.7-38.4	30.3-66.1	136.7-143.3	50.0-56.4			
Log-rank		0.189			0.083			

CI: Confidence interval; PRS: Post recurrence survival; OS: Overall survival; CR: Complete response; PR: Partial response; SD: Stationary disease; PD: Progression of disease.

Treatment and outcomes

The median time of post-recurrent survival was 6.7 mo (range 1.2-90 mo). Although surgical re-resection is a better option for recurrent disease, complete re-resection for recurrent disease was only performed for 6 patients. The median time of post-recurrence survival in these patients was 15.7 mo. Chemotherapy (doxorubicin〔adriamycin〕and dacarbazine〔DTIC〕) was administered to 5 patients with median survival of 3.7 mo. Twenty-two patients with recurrence were given supportive treatments such as analgesics or nutritional supplements (median survival of 5.9 mo). Re-resection significantly prolonged post-recurrence patient survival when compared with the patients treated

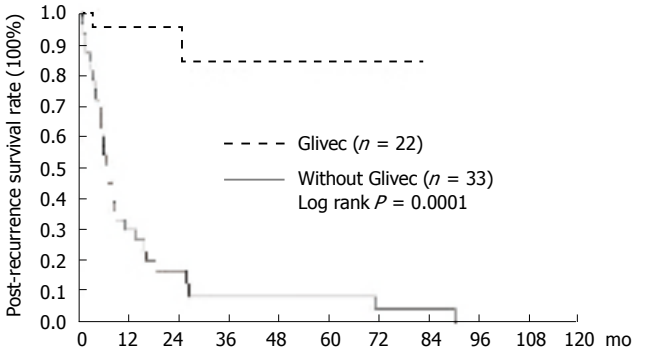


Figure 4 Post-recurrence survival rate of 55 small bowel GIST patients treated with and without Glivec.

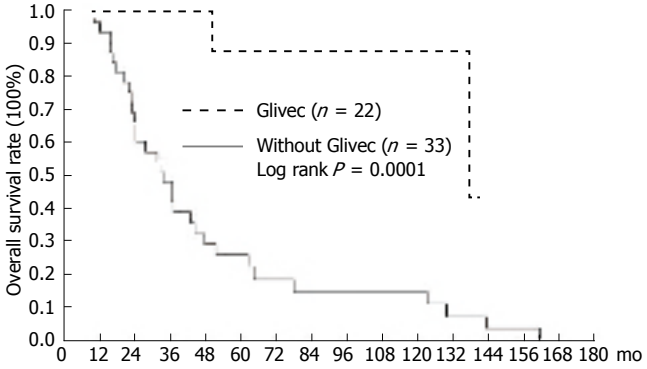


Figure 5 Overall survival rate of 55 small bowel GIST patients treated with or without Glivec.

with chemotherapy and conservative treatment (P = 0.00481), but did not improve overall survival rates (P = 0.1887). Glivec was administered to patients with unresectable or metastatic (advanced) small bowel GISTs from 2000. Twenty-two patients with advanced stages of the disease were given 400 mg Glivec per day. Advanced small bowel GIST patients treated with Glivec had significantly longer post-recurrence survival and overall survival than those not treated with Glivec (P = 0.0001) (Table 2) (Figures 4 and 5). The median follow-up time was 36.3 mo, range 8 mo to 142.4 mo. Although edema was common (14/22; 60.7%), therapy with Glivec was well tolerated. Overall, 15 patients (68.2%) had a partial response (PR), 4 stationary disease (SD) (18.2 %), 3 progressive disease (PD) (13.6%) (Table 3).

Spectrum of mutations in 22 small bowel GIST patients

Tumor specimens suitable for genetic analysis were available from 19 (86.4%) of the 22 small bowel GIST patients (Figures 3A-D and Table 4). Overall, 16 (72.7%) of the 22 examined GISTs had activated mutations of kit exons 9, 11, 13, or exon 17. No GIST had an activated mutation in more than one kit. Three of 19 GISTs expressed exon 9 mutation, 13 had exon 11 mutation, and three patients had no mutation of kit. No PDGFRA mutant isoforms were found. All the three GISTs had kit exon 9 mutation and displayed in-frame duplication of nucleotides, resulting in insertion of alanine (A) and tyrosine (Y) at condons 502 and 503. Among 13 GIST

Table 3 Antitumor response and mutation status of 22 advanced and metastatic small bowel GIST patients treated with imatinib meslyate

	CR	PR	SD	PD	*P*
Exon 9 (*n* = 3)	0	2	0	1	0.486
Exon 11(*n* = 13)	0	9	2	2	
No mutation (*n* = 3)	0	3	0	0	
Insufficient specimen (*n* = 3)	0	1	2	0	
	Exon 9 (*n* = 3)		Exon 11 (*n* = 13)		1.0
PR, *n* (%)	2 (66.7)		9 (69.2)		
SD + PD, *n* (%)	1 (33.3)		4 (30.8)		

PR: Partial response; SD: Stationary disease; PD: Progression of disease.

Table 4 Mutation patterns of 22 advanced and metastatic small bowel GIST patients treated with imatinib meslyate

Case (*n*)	Mutation pattern
2	Deletion mutation at codon 551-556 in exon 11
4	Deletion mutation at codon 557-561 in exon 11
8	Deletion mutation at codon 550-5 in exon 11
12	Deletion mutation at codon 563-576 in exon 11
13	Deletion mutation at codon 575-576 in exon 11
19	Deletion mutation at codon 569-575 in the exon 11
24	Deletion mutation at of codon 555-572 in exon 11
1	Deletion and insertion mutations at codons 563-572 in exon 11
15	Deletion and insertion mutations at codons 557-8 in the exon 11
22	Deletion and insertion mutations at codon 563-574 in exon 11
3	Missense mutation at codon 556 in exon 11
18	Missense mutation at codon 576 in the exon 11
21	Missense mutation at codon 557 in exon 11
7	Insertion mutation at codon 502-3 AY(alanine and tyrosin) in exon 9
9	Insertion mutation at codon 502-3 AY(alanine and tyrosin) in exon 9
17	Insertion mutation at codon 502-3 AY(alanine and tyrosin) in exon 9
10	No mutation
14	No mutation
20	No mutation

patients who had kit exon 11 mutations, 3 exhibited insertion/deletion, 7 showed deletion, and 3 demonstrated missense mutations (Table 4). Three of 19 tested GIST patients had no detectable kit mutation (kit wild-type) and no PDGFRA mutation. In 13 patients with GISTs harboring kit exon 11 mutations, the partial response rate (PR) was 69.3%, whereas two of three patients with tumors containing an exon 9 kit mutation had an ORR rate of 66.7% (Table 3, not significant).

DISCUSSION

The demographic features, tumor locations, and clinical features for Asian small bowel GIST patients are similar to those identified in other series[3,10,20-22]. Since the symptoms of small bowel GIST are vague, early diagnosis is not always easy and tumor sizes are usually large at diagnosis[3,10,20-22]. Before the introduction of Glivec, poor responses to radiation and chemotherapy have made surgery the only realistic treatment to cure the primary lesion[3,10,23-25]. A substantial number of patients with initial resection of GISTs eventually experience recurrence. The liver is the most frequent site for recurrence. There has been no effective treatment for advanced GISTs and the outlook for patients is extremely poor. As shown in this study, patients with recurrence who underwent further surgery with curative intention had a significant longer post-recurrence survival than those treated with chemotherapy or conservative treatment. However, the overall survival rates for both groups were similar. After treatment with Glivec, advanced small bowel GIST patients had significant longer post-recurrence and overall survival.

Therapeutic responses to targeted inhibition of activated tyrosine kinases have been demonstrated in certain types of leukemia, sarcoma, and breast cancer[19]. The mechanisms of kinase activation vary considerably among these cancers, but study of the influence of these mechanisms on drug response has been limited[19]. GISTs in particular, present a variety of genomic mutations across two different receptor tyrosine kinase genes. The kit or PDGFRA mutation in Asian clinically advanced small bowel GIST patients was examined in this study. Most small bowel GISTs expressed kit mutation (16/19, 84.2%). The kit kinase oncopproteins were intrinsically sensitive to Glivec, accounting for the excellent overall clinical response to Glivec, and coincident with results obtained by Heinrich *et al*[19]. Similar to that in report by demetri *et al*[16], the PR rate for Glivec in this study was 68.2%.

Gain-of-function mutations of PDGFRA has been recently discovered in GISTs[26] and studies have reported that PDGFRA and kit mutations are mutually exclusive[19]. However, no PDGFRA mutations were detectable in this series. More cases should be studied to clarify the racial differences regarding the incidence of PDGFRA mutation in GIST patients. A subset of GIST tumors in this study lacked detectable kit or PDGFRA mutations. Although such GISTs lack apparent genomic mutations, they can express phosphorylated kit or PDGFRA proteins that likely contribute to tumor proliferation or survival[19]. Contrary to the observation of Heinrich *et al*[26], GISTs lacking a detectable kinase mutation had a similar PR rate for Glivec to tumor with an exon 11 mutation or an exon 9 mutation (100% *vs* 69.2% *vs* 66.7%, $P = 0.527$).

In contrast to Heinrich *et al*[26], the PR rate did not differ between the groups of patients whose GISTs had kit exon 9 and exon 11 mutation (66.7% *vs* 69.2%), suggesting that the kit oncoproteins encoded by exons 9 and 11 are equally sensitive to Glivec *in vitro*[19].

In conclusion, Glivec significantly prolongs the post-recurrence and overall survival of Asian patients with advanced GISTs. Glivec induces a sustained objective response in more than half of Asian patients with advanced small bowel GISTs. Activated mutations of kit exon 11 are detectable in the vast majority of GISTs. There is no difference in the PR rate for patients whose GISTs have KIT exon 9 and exon 11 mutations.

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RAPID COMMUNICATION

Loss of heterozygosity and microsatellite instabilities of fragile histidine triad gene in gastric carcinoma

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Abstract

AIM: To detect the loss of heterozygosity (LOH) and microsatellite instabilities (MSI) of fragile histidine triad (FHIT) gene in gastric carcinoma and to study their association with the clinical pathological characteristics of gastric carcinoma.

METHODS: LOH and MSI of FHIT gene were detected at four microsatellite loci D3Sl3H, D3S4l03, D3Sl48l and D3S1234 using PCR in matched normal and cancerous tissues from 50 patients with primary gastric cancer.

RESULTS: The average frequency of LOH and MSI of FHIT gene in gastric cancer was 32.4% and 26.4% respectively. LOH and MSI of FHIT gene in gastric cancer had no association with histological, Borrmann, and Lauren's classification. LOH of FHIT gene in gastric cancer was related to invasive depth. The frequency of FHIT LOH in gastric cancer with serosa-penetration was obviously higher than that in gastric cancer without serosa-penetration (73.5% *vs* 37.5%, $P < 0.05$). MSI of FHIT gene in gastric cancer was associated with the lymph node metastasis. The frequency of MSI in gastric cancer without lymph node metastasis was significantly higher than that in gastric cancer with lymph node metastasis (66.7% *vs* 34.3%, $P < 0.05$).

CONCLUSION: LOH of FHIT gene is correlated with invasive depth of gastric carcinoma. MSI of FHIT gene is correlated with lymph node metastases. LOH and MSI of FHIT gene play an important role in carcinogenesis of gastric cancer.

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Key words: Gastric cancer; FHIT gene; Microsatellite instabilities

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INTRODUCTION

Gastric carcinoma is the most frequent malignancy and the leading cause of cancer-related deaths in China[1-4]. The specific oncogenes and tumor suppressor genes correlated with gastric cancer have not been found. In recent years, many genes have been found to be related to gastric carcinogenesis, such as fragile histidine triad gene (FHIT), RUNX3 and PTEN, among which FHIT gene is a tumor suppressor gene[5] which is located in chromatosome 3p14.2, and comprises 10 exons. Exons 1-4 and 10 are non-coding exons, and exons 5-9 are coding exons. Recent studies indicate that FHIT abnormality mostly results from loss of exons 5 and 8[6-9]. Exon 5 contains the start codon (ATG) of opening read frame (ORF), exon 8 contains core unit of coding histidine triplet. Chromosome 3p14.2 containing the common apaphidicolin-induced fragile site (FRA3B)[10-13], is the non-randomly fragmental or fissured site by its spontaneous or induced actions, and susceptible to chromosome loss and rearrangement because of the frequent fragmentation or fissure, which might be related to tumor-genesis. In this study, we detected the loss of heterozygosity (LOH) and microsatellite instabilities (MSI) of microsatellite alleles close to FHIT gene in 50 cases of gastric cancer.

MATERIALS AND METHODS

Specimens

All specimens of gastric cancerous and matched distal normal mucosal tissues were surgically resected at No.1 Hospital of China Medical University without preoperative chemotherapy and radiotherapy. Specimens were put immediately into liquid nitrogen after removal, stored at -70℃, and classified respectively by Borrmann, WHO histological classification, Lauren classification, invasive depth and lymph node metastasis, *etc.*

Table 1 Primer sequences of FHIT gene at different sites

MSM		Sequence	Product lengh
D3S1300	F	5′-ATAAACACTACAAAGGAAC-3′	49 bp
	R	5′-TATCTTAAAATTGCACCC-3′	
D3S1234	F	5′-ACGGTGGACGAGAAGAAC-3′	612 bp
	R	5′-TTTGAACAAGAAGTGGCATA-3′	
D3S1481	F	5′-CTGTGGGTTGTCTGTTTA-3′	489 bp
	R	5′-CTGGAGGCATCTCATTAC-3′	
D3S4103	F	5′-TGGTTTGGTGCTATTCTC-3′	226 bp
	R	5′-TTATTCGGCTCATTGTTC-3′	

MSM : Microsatellite marker.

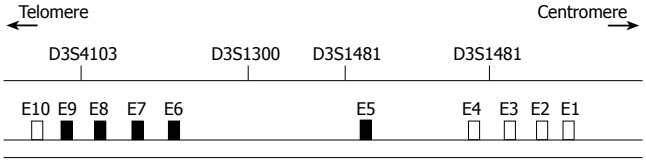


Figure 1 FHIT gene cDNA at different sites.

DNA extraction

DNA was extracted from the frozen gastric cancerous and normal mucosal tissues by standard phenol-chloroform method[3,4], dissolved and stored in TE buffer. Finally, purity and concentration of the extracted DNA were analyzed by gel electrophresis and ultraviolet spectrophotometry.

Microsatellite marker selection and PCR amplification

Four microsatellite marker sites (D3S1300, D3S1234, D3S4103 and D3S1481) in chromosome 3p14.2 were selected to detect LOH and MSI of FHIT gene. PCR primers (sequences in Table 1 and sites in Figure 1) were synthesized by Beijing AoKe Biology Company. PCR amplification was carried out in a final volume of 50 μL containing 50 ng DNA, 0.5 μmol/L of each primer, 200 μmol/L of each dNTP, and 1U Taq DNA polymerase (TaKaRa Ex Taq™). The amplification conditions were an initial denaturation at 94℃ for 3 min, followed by 30 cycles at 94℃ for 30 s, at 57℃ for 1min, at 72℃ for 40 s, and a final extension at 72℃ for 5 min.

Polyacrylamide gel electrophoresis (PAGE)

The amplified PCR products were denatured at 95℃ for 5 min and run on 8% denaturation polyacrylamide gel (1 × TBE buffer, circulatory water) at a voltage 500 V, power 45 W for 3.5 h. Silver staining was performed as previously described [14].

Determination of FHIT LOH and MSI

Genomic allele with heterozygote was considered as target of LOH analysis information. LOH was defined as complete loss or up to 50% decreased relative density of silver staining bands of PCR products in primary gastric cancerous tissues compared to distal normal mucosal tissues, and MSI was defined as the increased or mobile bands of PCR products of gastric cancerous tissues compared to normal tissues.

Table 2 FHIT gene LOH and MSI at different microsatellite sites *n* (%)

MSM	*n*	AIT	MSI	LOH
D3S1300	50	38 (76.0)	14 (36.8)	10 (26.3)
D3S1234	50	42 (80.4)	9 (21.4)	13 (31.0)
D3S1481	50	29 (54.5)	6 (20.7)	8 (27.6)
D3S4103	50	39 (74.5)	10 (25.6)	17 (43.6)

MSM: Microsatellite marker; AIT: Analysis information target; MSI: Microsatellite instabilities; LOH: Loss of heterozygosity.

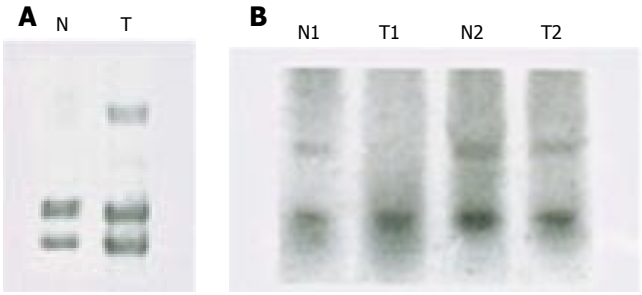


Figure 2 MSI (**A**) and LOH (**B**) at D3S4103. T: Gastric cancer tissues; N: Normal gastric mucosal tissues.

Statistic analysis

Sample rate between different groups was analyzed with chi square test. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of FHIT gene LOH and MSI in gastric cancers

The frequencies of LOH and MSI of FHIT gene in four microsatellite sites are shown in Table 2. The average frequency of FHIT gene LOH in gastric cancer was 32.4%, and the most frequent site was D3S4103, accounting for 43.6% (Figure 2A). All samples had different degrees of LOH at four sites. The average frequency of FHIT gene MSI in gastric cancer was 26.4%, the most frequent site was D3S1300, accounting for 36.8% (Figure 2B), and 11 cases (22.0%) had MSI at two or more microsatellite sites.

Relationship between FHIT gene LOH, MSI and clinicopathological characteristics of gastric cancer

There was no statistical relationship between FHIT gene LOH and Borrmann's, WHO's, Lauren's classification and lymph node metastasis of gastric cancer ($P > 0.05$), while FHIT gene LOH was significantly associated with invasive depth of gastric cancer. LOH frequency in gastric cancers penetrating serosa was significantly higher than that in gastric cancers not penetrating serosa ($P < 0.05$). There was no statistical relationship between FHIT gene MSI and Bormann's, WHO's, Lauren's classification and invasive depth of gastric cancer ($P > 0.05$), while FHIT gene MSI was significantly associated with lymph node metastasis

Table 3 Relationship between FHIT gene LOH, MSI and clinicopathological characteristics of gastric cancer *n* (%)

	n	MSI [1]	LOH [2]
Histological type			
pap.ade & wel.ade	6	3 (50)	4 (66.7)
mod.ade	9	5 (55.6)	5 (55.6)
por.ade	26	13 (50.0)	17 (65.4)
muc.ade	7	3 (42.9)	3 (42.9)
SRC	2	1 (50.0)	1 (50.0)
Lauren			
Diffusive type	35	17 (48.6)	21 (60.0)
Intestinal type	15	8 (53.3)	9 (60.0)
Bormann			
I	5	3 (60.0)	3 (60.0)
II	10	6 (60.0)	7 (70.0)
III	15	7 (46.7)	10 (66.7)
IV	10	5 (50.0)	7 (70.0)
Invasive depth			
without serosa penetration	16	7 (43.8)	6 (37.5)
with serosa penetration	34	18 (52.9)	25 (73.5)[a]
LN metastasis			
+	35	12 (34.3)	21 (60.0)
-	15	10 (66.7)[a]	9 (60.0)

[1] MSI occurring at least one microsatellite site; [2] LOH occurring at least one microsatellite site; [a]$P < 0.05$ *vs* without serosa penetration and with LN metastasis.

of gastric cancer. MSI frequency in gastric cancers with lymph node metastasis was significantly higher than that in gastric cancers without lymph node metastasis ($P < 0.05$, Table 3).

DISCUSSION

FHIT gene fragile site containing instable repeated sequence of tris-nucleotide (CGG or CCG) or bi-nucleotide (AT), is subjected to easy breakage because of its susceptibility to external environment carcinogen and mutagen. There are many Alu repeated sequences in proximal telomere of fragile site FRA3B. Susceptible fragile site has highly instable repeated sequence $(TAA)_{15}$[11,12,15-20]. It can be inferred that under the action of external carcinogen, breakage and crevice may occur at FRA3B site, following DNA abnormal repair and rearrangement, thus making complex diversity of FHIT gene[21-24].

Our study showed that the average frequeny of FHIT gene LOH and MSI was 32.4% and 26.4% respectively in gastric cancer. There was no significant discrepancy between different microsatellite sites. The most frequent site of LOH and MSI was D3S4103 (43.6%) and D3S1300 (36.8%) respectively located in the first coding exon 5 near the FHIT gene, suggesting that FHIT gene loss is common in gastric cancer. Moreover, many cases had both LOH and MSI at two or more sites, suggesting that variant range of FHIT gene is broader. We did not discover that FHIT gene LOH was associated with Borrmann, histological and Lauren's classification as well as lymph node metastasis. But significant relationship was found between FHIT LOH and invasive depth of gastri cancer. Huiping *et al*[6] reported that gastric cancer is concomitant with frequent FHIT gene LOH and MSI (84% and 27% respectively), and loss of FHIT protein is closely associated with lymph node metastasis. Cappuzzi *et al*[7] also reported that loss of FHIT protein is positively correlated with clinicopathological stage and histological grade of gastric cancer, but negatively with poor prognosis. Noguchi *et al*[25] showed that LOH of FHIT gene is not related to progression and prognosis of gastric cancer.

There were no significant differences in FHIT gene LOH and MSI among various gastric cancers, suggesting that FHIT gene loss might not be associated with different gastric cancer. FHIT gene LOH was significantly associated with invasive depth of gastric cancer. LOH frequency in gastric cancers penetrating serosa was significantly higher than that in gastric cancer not penetrating serosa, suggesting that FHIT gene LOH is correlated with infiltration and diffusion of gastric cancer. FHIT gene MSI was significantly associated with lymph node metastasis of gastric cancer. MSI frequency in gastric cancers with lymph node metastasis was significantly higher than that in gastric cancers without lymph node metastasis, suggesting that FHIT gene mainly participates in early gastric carcinogenesis. The mechanism of down-regulated expression of FHIT post-transcription needs further study.

In conclusion, FHIT LOH and MSI reflect the genomic instability of gastric cancer and play an important role in gastric carcinogenesis. Abnormal FHIT gene may provide some new clues to malignant biological behavior and molecular mechanism of gastric carcinogenesis.

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RAPID COMMUNICATION

Purification and characterization of α-L-fucosidase from human primary hepatocarcinoma tissue

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Abstract

AIM: To purify and characterize α-L-fucosidase from human liver cancer tissue and to detect the localization of α-L-fucosidase in tumor tissue.

METHODS: Cation exchange chromatography on CM-52 and ultrafiltration were used to separate α-L-fucosidase (AFU) from crude extract of liver cancer tissue. 4-methylumbelliferyl-α-L-fucopyranoside was used as a fluorescent substrate to quantify the purified AFU activity in each step. A polyclonal antibody (pAb) against the purified AFU was obtained by anion exchange chromatography on DEAE-52 after ammonium sulfate fractionation and ultrafiltration. Immuohistochemical staining was used to observe the expression of AFU in malignant and adjacent liver tissues.

RESULTS: Human α-L-fucosidase was purified 74–fold to apparent homogeneity with 15% yield. SDS-PAGE indicated the presence of one subunit of molecular weight of 55 Ku. The specific activity of AFU in pooled fraction by chromatography was 10085 IU/mg. Western blot analysis indicated that the pAb could recognize one protein band of molecular weight of 55 Ku. The expression of AFU was observed in cytoplasm membrane of liver cancer tissue but not in that of adjacent tissue.

CONCLUSION: The purified α-L-fucosidase from primary hepatocarcinoma (PHC) is different in its properties from α-L-fucosidase in human other organs. The polyclonal antibody prepared in this experiment can be applied to the diagnosis of PHC.

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Key words: α-L-fucosidase; Primary hepatocarcinoma; Polyclonal antibody

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INTRODUCTION

α-L-fucosidase (AFU) is a glycosidase primarily found in lysosome and involved in the degradation of a variety of fucose-containing fucoglycoconjugates[1,2]. The necessity and importance of AFU (α-L-fucoside fucohydrolase, EC 3.2.1.51) are demonstrated by deficiency of its enzymatic activity leading to fatal neurovisceral storage disorder designated fuocosidosis characterized by mental and motor retardation [3]. The alterations of AFU catalytic activity in human cells, tissues and body fluids have a diagnostic value for human tumors such as primary hepatocarcinoma (PHC)[4-8], colorectal cancer[9-11], ovarian cancer[12].The deficiency of AFU activity in female sera is probably a hereditary condition related to higher risk of ovarian cancer[2,12]. The persistently elevated AFU level in sera of patients with liver cirrhosis contributes to early detection of PHC[5]. In addition, information of the structure and biochemical characteristics of AFU helps to understand the pathogenesis of fuocosidosis on molecular level and to discover therapeutic approaches for the disease[1,13]. In the present investigation, we described an improved purification procedure for AFU by ion exchange chromatography and immunochemical studies using polyclonal antibody against the homogeneous enzyme from human PHC tissue.

MATERIALS AND METHODS

Materials

Liver specimens were obtained from patients with PHC who underwent partial hepatectomy at Tongji Hospital. The procedure conformed to the local ethical guidelines. Informed consents were obtained from the patients or their relatives. The study protocol followed the ethical guidelines of the Helsinki Declaration of 1975. Rabbits used for antiserum production procedure received human care. All procedures were performed at 4℃ unless otherwise specified. Substrate 4–methylumbelliferyl-α–L–fucopyranoside (4-MU-Fuc) and standard product 4–methylumbelliferone (4-MU) were purchased from

Sigma. CM-52 and DEAE-52 cellulose resin were purchased from Whatman, USA.

Enzyme assay

AFU activity was assayed using 4-MU-Fuc as previously described with a minor modification[14-15]. Thirty μL aliquots of enzyme preparation was incubated at 37℃ for 30 min with 150 μL of 0.2 mol/L citrate buffer (pH 5.0) containing 60 nmol/L 4-MU-Fuc. The reaction was terminated by addition of 4 mL of 0.2 mol/L K_2CO_3 (pH 10.0). The fluorescent absorbance was read on a HITACHI F-4500 spectrophotometer with excitation at 365 nm and emission peak at 460 nm. Readings were corrected by subtracting tissue and substrate blanks. One unit of enzyme activity was defined as the amount generating 1 nmol of product 4-MU per min at 37℃. Protein concentration was determined by the traditional method of Bradford[16] using Coomassie brilliant blue G-250, following the standard assay and microassay with a protein concentration assay kit (Nanjing, China) using bovine serum albumin as standard protein. To quantify AFU activity in each purification step, we used a modified procedure to determine enzyme activity which assays the activity of β-galactose and generates a standard curve of known product concentration[17].

Extraction and concentration of enzymes

Crude extract of PHC tissue was prepared as previously described[18] with certain modifications. Portions of human liver from patients with PHC diagnosed by pathological examination were washed in distilled water and cut into small sections, stored in 30% glycerol at -70℃ until use. About 25 g of liver tissue (wet weight) was cut into small blocks within 0.05 mol/L cold sodium acetate pH 5.0 (10 mL per gram of tissue, wet weight). The blocks were then homogenized in a glass homogenizer, heated to 37℃ for 1 h to release the enzyme. The pH was then immediately adjusted to 5.0 with glacial acetic acid. The mixture was heated to 60℃ for 10 min before it was cooled to room temperature on ice and then centrifuged at 0℃ for 30 min at 35 000 r/min. The precipitate was discarded and supernatant pH was adjusted to 5.0 with 15 N NH_4OH. Solid ammonium sulfate was added to the supernatant to 35% saturation. The solution was allowed to stand at 4℃ for 4 h, and then centrifuged at 0℃ for 30 min at 29000 r/min. The precipitate was discarded, and the supernatant was saturated to 50% ammonium sulfate by addition of solid ammonium sulfate and allowed to stand at 4℃ for 20 h, then centrifuged at 0℃ for 30 min at 29000 r/min. The precipitate was dissolved in 4.0 mL of 0.1mol/L sodium citrate (pH 5.0), 0.02% in NaN_3 to yield 35%-50% ammonium sulfate fractions. The supernatant was saturated to 60% of ammonium sulfate by addition of solid ammonium sulfate and allowed to stand at 4℃ for 20 h, then centrifuged at 0℃ for 30 min at 29 000 r/min. The precipitate was dissolved in 2.0 mL of 0.1mol/L sodium citrate (pH 5.0), 0.02% in NaN_3 to yield 50%-60 % ammonium sulfate fractions. The 35%-50% and 50%-60% ammonium sulfate fractions were stored at -20℃ for chromatography.

Chromatographic methods

Further fractionations of the 35%-50% and 50%-60% ammonium sulfate fractions were obtained by chromatography on CM-cellulose (Whatman CM-52). To keep the stability of enzyme activity as possible the whole procedure was performed at 4℃. The chromatography column (2.5 cm × 35 cm) was prepared in 0.011 mol/L citric acid-NaOH (pH 4.5), 0.02 % in NaN_3 as previously described[18]. Details are given in Figure1.

Molecular weight determination of purified AFU

The presence of subunits was determined in 12 % polyacrylamide gels (8 cm × 7.3 cm, 0.75 mm) containing 0.1% SDS as previously described[19]. Briefly, 5% stacking and 12 % running gels were run at room temperature using 25 mmol/L Tris-HCl, 0.2 mol/L glycine buffer (pH 8.3) containing 0.1% SDS for 30-40 min at 80 V and 1-1.5 h at 100 V respectively. AFU samples (20-25 μg/lane) were subjected to electrophoresis after treated with 2% SDS, 5% 2-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue in 60 mmol/L Tris-HCl (pH 6.8) in boiling water bath for 3 min. Prestained molecular mass standards (Fermentas, SM0671) were used and stained at the same condition. The gels were stained as previously described[20] with 0.25% Coomassie brilliant blue R-250 in glacial acetic acid: methanol: distilled water (10:45:45, v/v/v) for 3 h, then distained with the mixture of glacial acetic acid : methanol: distilled water (10:45:45, v/v/v) without Coomassie brilliant blue R-250 for 1 h, 20 min per time.

Immunization of animals

All procedures were performed as previously described[21] unless otherwise specified. Antiserum against AFU was produced by immunizing rabbits with 0.5 mg of purified AFU in equal volume of Freund's complete adjuvant, and 5 mL serum was collected before immunization as a normal control. The emulsion was injected subcutaneously at multiple sites on the rabbit metapodium palm. After 2 wk, the rabbits were injected with 0.5 mg purified AFU in Freund's incomplete adjuvant intramuscularly in each caudal thigh muscle. After 3 wk, the rabbits were given 0.2, 0.2, 0.4 mg of purified AFU respectively in a week at different sites on ear central artery for booster injection. A blood sample from ear central artery was collected for determination of the tilter after the second immunization. One week after the last booster injection, the rabbits were exsanguinated *via* cardiac puncture under general anaesthesia using diethyl ether.

Purification of α-L-fucosidase IgG

Blood was stood at room temperature for 1 h at 4℃ overnight, then centrifuged at 13000 r/min for 30 min at 4℃.The resulting pellet was discarded with the supernatant collected. The whole serum was precipitated with saturated ammonium sulfate to a final saturation of 33%, then desalted with Amicon Ultra-15 PLGC centrifugal filter unit (Millipore, NMWL, 10 KDa) (http://www.millipore.com/catalogue.nsf/docs/C7715).

Anion exchange chromatography

The desalted antiserum was added to anion exchange

Table 1 Purification of AFU from human PHC tissue

Fraction	Protein (mg)	AFU (units)	Specific activity (units/mg protein)	Purification fold	Yield (%)
Homogenate and incubate at 37℃	878.4	120024	137		
35%-50% $(NH_4)_2SO_4$ precipitation	126.55	95352	753	5	79
50%-60% $(NH_4)_2SO_4$ precipitation	46.55	16420	353	3	14
Ultrafiltration fraction					
35%-50% $(NH_4)_2SO_4$ precipitation	22	46676	2122	16	39
50%-60% $(NH_4)_2SO_4$ precipitation	5.6	6835	1221	9	6
CM-cellulose fraction	1.82	18354	10085	74	15

column (DEAE-52, Whatman) pre-balanced with 0.005mol/L balancing buffer, pH 8.6, Tris-PO_4 and stood at 4℃ for 30 min. Fractionations were eluted using 0.055 mol/L (pH 6.0) and 0.5 mol/L (pH5.1) Tris-PO_4 by a stepwise developing method, and pooled according to their protein content determined by absorbance of optical density at 280 nm. Because of instability of the purified antibody, chromatography should be carried out at 4℃. Pooled fractionations from DEAE-52 were adjusted to pH 6.4 with 10mol/L NaOH and stored at 4℃ to preserve their activity.

Western blot analysis

The proteins from slab gels were electrotransferred to 0.2 μm-pore-size nitrocellulose membrane (Schleicher and Schuell, Keene, NH) in 48 mmol/L Tirs/HCl transferring buffer containing 39 mmol/L glycine, 0.037% SDS, 20% methanol, at 4℃ and 350 mA for 70 min. A portion of nitrocellulose was stained as previously described[22] for 5 min with a working solution of 10-fold dilution of 2% ponceau S, 30% trichloracetic acid (TCA), 30% salicylsulfonic acid, solved in distilled water to 100 mL total volume, then destained vigorously in TBST with shaking until the ponceau S was washed off. The remainder of nitrocellulose was blocked for 2 h under constant shaking at room temperature in 5% non-fat dry milk dissolved in Tris-buffered saline-Tween-20 (TBST) containing 10 mmol/L Tirs/HCl (pH 7.5), 0.15 mol/L NaCl and 0.05% Tween-20. The membrane was incubated overnight at 4℃ in 100-fold dilution of immunoglobulin G (IgG) fraction of anti-AFU polyclonal antibody, and washed three times with TBST under constant shaking for 1 h, 20 min per time. The membrane was incubated with the secondary antibody at room temperature for 2 h under constant shaking, 5000-fold dilution of horseradish peroxide-conjugated immunoPure goat anti-rabbit IgG antibody [IgG (H+L),blotting grade; Pierce]. After three more 20 min washes with shaking (10 mmol/L-Tirs/HCl buffer, pH 7.4), development was accomplished by enhanced chemiluminescence (ECL) for 1 min following the manufacturer's instructions (Pierce), and the membrane was exposed to Kodak X-ray film. Exposure time was determined on the basis of signals generated by the reaction between membrane and mixture solution from ECL kit. The results were obtained through Kodak medical X-ray processor 102 (Eastman Kodak, Rochester, USA).

Streptavidin-peroxidase-biotin (SP) immunohistochemistry

The samples were incubated with the primary antibody against AFU (1:50, purified polyclonal, diluted in PBS) at 4℃ overnight. SP-immunohistochemistry (SP-IHC) was performed according to the manufacture's instructions (Zhong Shan Ltd Co, China) for SP kit. Sections were stained with 3, 3'-diaminobenzidine (DAB) and counterstained with haematoxylin for visualization of nuclei. In negative controls, phosphate-buffered saline (PBS) was chosen as the primary antibody instead of anti-human AFU polyclonal antibody.

RESULTS

Purification of AFU

An effective procedure was developed for the purification of AFU from human primary hepatocarcinoma tissue. The process included homogenization, high speed centrifugation, ammonium sulfate precipitation, ultrafiltration and cation exchange chromatography. The results are summarized in Table1. This procedure typically resulted in a purification of 74-fold with a very high specific activity of 10085 (nmol.min/mg) protein in 15% yield.

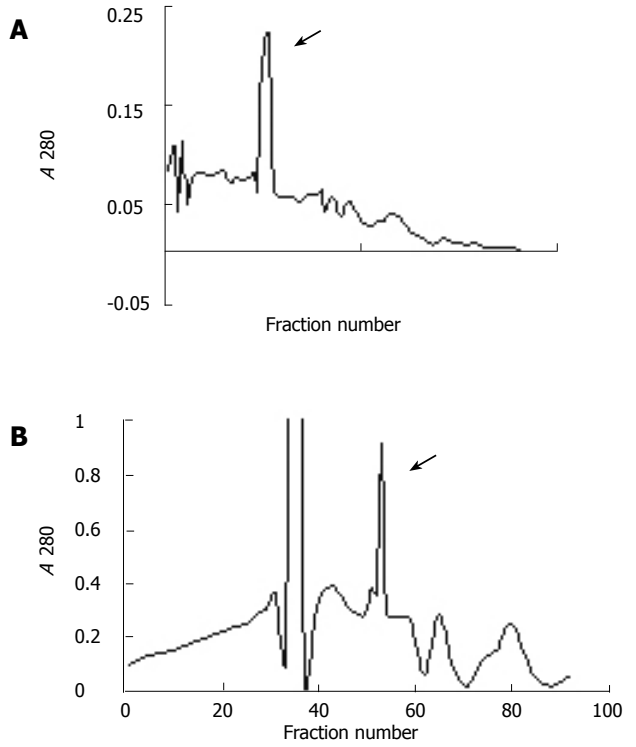
The stability of purified AFU was evaluated following storage at -20℃, 4℃ and 20℃ (pH 5.0) in the presence of sample buffer respectively. Samples in the frozen condition retained 100% of enzyme activity for at least two months. While samples in refrigerated condition showed slow but progressive decrease of enzyme activity with about 50%-80% of initial activity after two-month storage. Observations also exhibited that 100% enzyme activity remained in aseptic condition at room temperature within two days.

The elution schemes of two fractionations by chromatography are shown in Figure 1.

Standard curve of AFU

To quantify AFU activity in each purification step, we modified a procedure to assay the activity of β-galactose[17]. Using this method, enzyme activity in unknown samples could be compared according to a standard curve.

The absorbance of known concentrations of commercially available products generated at excitation wavelength 365 nm and emission wavelength 460 nm was



Figurge 1 Elution of fractionations on cation exchange. **A:** Chromatography of the ultrafiltration fraction of 35% to 50% $(NH_4)_2SO_4$ ammonium sulfate precipitation (22 mg of protein) on CM-cellulose. The procedure was performed with a column (2.6 cm × 30 cm) at a flow rate of 65 to 75 mL per hour. About 5 mL fractions was pooled. The column was developed initially with 750mL of 0.011 M citric acid-NaOH (pH 4.5), 0.02% NaN_3, and then a stepwise dilution over 400 mL from 0.05, 0.10, 0.15 to 0.25 mol/L NaCl was used to accomplish the development; **B:** Chromatography of the ultrafiltration fraction of 50% to 60% $(NH_4)_2SO_4$ ammonium sulfate precipitation (5.6 mg of protein) on CM-cellulose. The arrows indiente the elution peak of the interest protein.

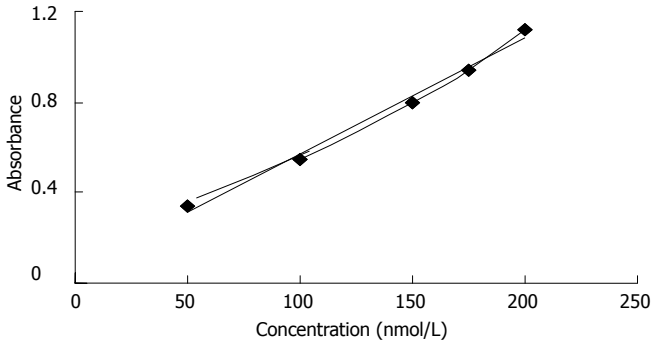


Figure 2 Stand curve of AFU. Solid line represents an artifact line generated by the Microsoft excel™ and dot line represents the practical values in linear range.

shown using HITACHI fluorescence spectrophotometer (Figure 2).

Purity of AFU in human liver cancer tissue

The migration pattern of crude extracts and purified AFU in denaturing polyacrylamide gel electrophoresis is shown in Figure 3. The SDS-PAGE indicated the presence of one subunit of purified AFU at 55 Ku.

Purification and purity of AFU antiserum

Antiserum was obtained via cardiac puncture by high

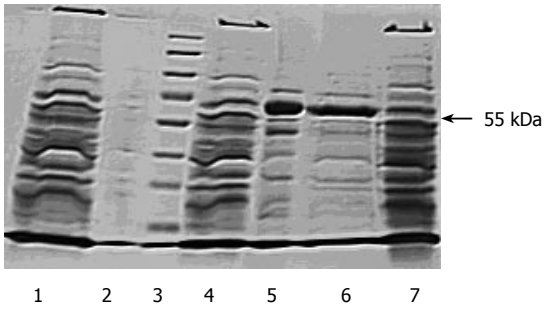


Figure 3 Electrophoretic analysis of AFU in crude and purified human liver. Lane 1: 50% to 60% $(NH_4)_2SO_4$ precipitation; lane 2: supernatant after high speed centrifugation; lane3: protein standards(Fermentas , prestained); lane 4: 35% to 50% $(NH_4)_2SO_4$ precipitation; lane 5: ultrafiltration fraction after 35% to 50% $(NH_4)_2SO_4$ precipitation; lane6: CM-cellulose fraction; lane 7: Homogenate and incubate at 37℃.

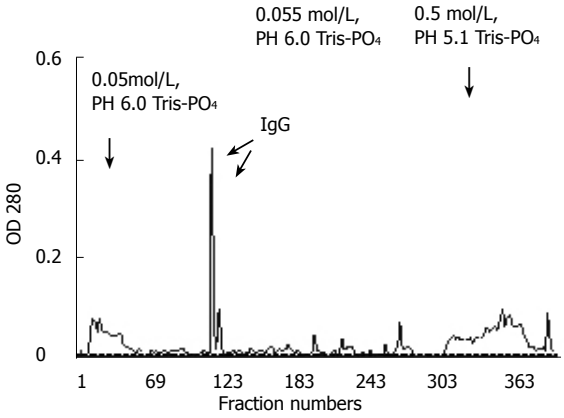


Figure 4 Anion exchange chromatography of crude antiserum against human PHCα-L-fucosidase using a column (2.6 cm × 30 cm) of DEAE-cellulose. The column was eluted as described in Materials and methods. The arrows indicate the applied buffers and the emerged elution peak of IgG.

speed centrifugation, ammonium sulfate precipitation, ultrafiltration and anion exchange chromatography on DEAE-cellulose. The elution profile of anti-serum from anion exchange column is depicted in Figure 4. The curve showed an elution peak of IgG fraction of antiserum against AFU around the 115th collecting tube. SDS-PAGE analysis illustrated the single subunit of 55 kDa of purified IgG and appeared to be highly purified.

Western-blot analysis of human liver AFU

Western blot analysis indicated that purified AFU antiserum recognized the 55 kDa band of the enzyme compared with immunized rabbit serum used as a control. The density of bands seemed to become thicker with prolonged exposure time (Figure 5).

Location and distribution of AFU expression

Immunohistochemistry staining indicated that predominant expression of AFU could be seen primarily in plasma membrane and cytoplasm of primary hepatocarcinoma cells compared with adjacent tissue and negative control. This staining was not found in the negative control treated identically except for primary antibody against human liver AFU.

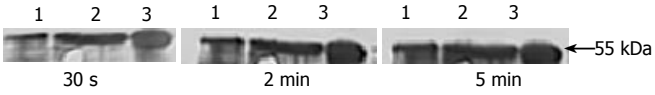


Figure 5 Western blot analysis showing that purified AFU could recognize a single subunit of 5 Ku (transverse arrow of the enzyme at 30s, 2 min, 5 min, respectively. Lane 1: purified AFU (5 μg) detected with a 10^3 dilution of antiserum; lane 2: purified AFU (10 μg) detected with a 5×10^2 dilution of antiserum; lane 3: purified AFU (5 μg) detected with a 5×10^2 dilution of antiserum.

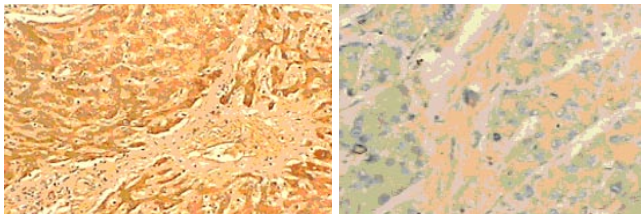


Figure 6 AFU expression in cytoplasm and/or cell membrane of PHC.

DISCUSSION

A number of investigations on α-L-fucosidase (EC 3.2.1.51) in different species have provided evidence for its importance in organism metabolism and diseases[1-3]. Although AFU is of considerable interest in human disease research because of fucosidosis, neurovisceral storage disease lacking of its enzymatic activity, many studies on its role in cancer research have been reported. To our knowledge, this is the first report of purification and characterization of human AFU from primary hepatocarcinoma tissue.

In this study, several criteria were chosen to purify and characterize AFU including ammonium sulfate precipitation, ultrafiltration, ion exchange chromatography, SDS-PAGE, Western blot, immunohistochemsitry staining and production of monospecific antibody against purified AFU. The results showed that this procedure was straightforward and successful for AFU purification. Contaminating glycosidases were removed during the course of each purification step and purified enzyme appeared to be homogeneity.

The molecular weight of purified enzyme determined by SDS-PAGE was a single subunit of 55 Ku, which is different from those reported previously[23-27]. These results may prove that purified AFU from PHC tissue is tetrameric, consisting of four identical subunits.

Western blot analysis showed enzyme of the 55 Ku subunit recognized by anti-AFU polyclonal antibody. None of the bands reacted with a preimmune serum (data not shown). This immunochemical recognition is consistent with previous studies on human colon AFU[11], one band of about 55 Ku was detected when incubated with an immunized serum, which is similar to the report of Johnson *et al*[28].

Immunohistochemistry staining showed predominant expression of AFU in plasma membrane and/or cytoplasm of primary hepatocarcinoma tissue, which is similar to previous results[29](Figure 6). The precise localization of AFU may suggest that expression level and specific site of AFU expression in primary hepatocarcinoma tissue can be a useful marker for early detection of PHC and a prognosis indicator of PHC development. It was reported that serum AFU activity is a useful marker for early detection of PHC particularly in α-fetoprotein -low or negative PHC patients[4-8,30]. However, the mechanism of elevated AFU in serum or tissues of patients with PHC is still obscure. One possible explanation is the increased synthesis and secretion of AFU by tumor cells[31]. Genetic studies suggest that three different phenotypes of AFU are dominated by two alleles exhibiting autosomal inheritance with a gene dosage effect. Individuals with high activity of AFU in serum are considered homozygous. In addition, sialic acid of glycoproteins determines the clearance rate in circulation and its transformation may cause retarded clearance of glycoproteins[32].

In conclusion, purified AFU and corresponding polyclonal antibody can be used as antigen-antibody candidates to detect PHC at early stage.

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CASE REPORT

Management of ischemic proctitis with severe rectal haemorrhage: A case report

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Abstract

Acute ischemic proctitis is a rare pathological condition. We report here a patient presented with massive rectal haemorrhage from a bleeding vessel superimposed on an underlying pathology of ischemic proctitis. This case report illustrates the difficulties in making the clinical distinction between ischemic proctitis and other pathological entities. We also discussed the beneficial role of arteriogram with embolotherapy as an effective therapeutic measure in the management of lower gastrointestinal bleeding. The literature on the subject is reviewed.

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Key words: Ischemic proctitis; Arteriogram; Embolization; Gastrointestinal bleeding

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INTRODUCTION

Ischemic proctitis is a rare pathological condition. Bharucha *et al*[1]reviewed all ischemic proctosigmoiditis cases between 1976 and 1991, and only identified 10 cases (6 acute and 4 chronic cases) out of 328 cases of colonic ischemia. Most ischemic proctitis reported was secondary to radiotherapy, previous vascular intervention, aorto-iliac surgery, vasculitis or myointimal hyperplasia of the mesenteric vein[1-5]. These interventions or pathologies can potentially compromise the arterial supply of the rectum, leading to ischemia. It is even less common for the occurrence of spontaneous ischemic proctitis, which accounted for less than 2% of all cases of ischemic colitis.

We present herein a patient with severe rectal haemorrhage from a bleeding vessel and a co-existing pathology of ischemic proctitis.

CASE REPORT

A 62-year-old male, with grade 2 primary hypertension (1999 WHO/ISH classification) and no other past medical history, first presented as an emergency with a 2-wk history of diarrhoea, colicky lower abdominal pain and proctalgia. Vital signs and abdominal examination were unremarkable but rectal examination revealed marked tenderness and fresh rectal bleeding.

Full blood count, CRP, urea and electrolytes were normal. Abdominal radiology and stool culture were negative. Examination under anaesthesia revealed marked inflammation and superficial ulceration in the rectum and distal sigmoid colon. These changes were in keeping with a presumptive diagnosis of inflammatory bowel disease. Whilst histology of rectal biopsy was awaited, the patient was commenced on steroid enemata and discharged with out-patient follow-up.

The patient was readmitted three days later with hypovolaemic shock secondary to profuse rectal bleeding. Following resuscitation and stabilization, an emergency mesenteric arteriogram was performed. Selective examination of the inferior mesenteric artery revealed for the first time vasculature abnormality arising from a branch of the superior rectal artery with early filling of a large vein, consistent with a radiological evidence of arterio-venous fistula (Figure 1). The artery was selectively catheterised and the feeding vessel was embolized with Gel foam pledgets (Figure 2). The rectal blood supply appeared to be intact after the embolization.

The patient rebled two days later and repeated arteriography demonstrated re-canalisation of the venous drainage. In view of the re-canalisation, segment of superior rectal artery was embolized with Gel foam pledgets and metallic coils. There was no radiological evidence of further extravasations following the second embolization (Figure 3). After the second radiological intervention, result of the rectal biopsy from the first admission was then available and demonstrated features consistent of ischemic proctitis.

The patient had another episode of rectal bleeding 48 h later. Flexible sigmoidoscopy showed a grossly ischemic

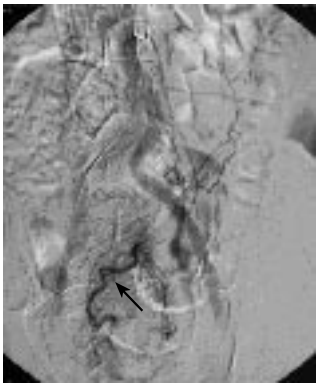


Figure 1 Arteriogram picture taken before the 1st embolization. Arrow demonstrates an abnormal vasculature.

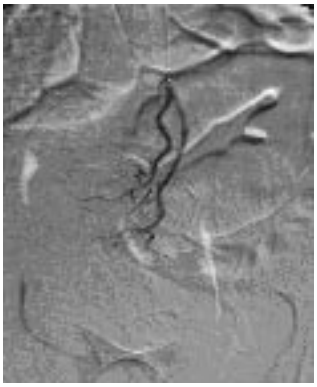


Figure 2 Arteriogram picture taken immediately after the 1st embolization. No abnormal vessels were seen.



Figure 3 Arteriogram picture taken after the 2nd embolization. Arrow demonstrates the Gel foam pledget and metallic coil at the superior rectal artery.

mucosa with numerous vertical linear ulcers. The segment of the affected rectum was well demarcated. A low Hartmann's procedure was successfully carried out with the preservation of the anal sphincter complex. Histology of the resected rectum demonstrated broad areas of necrosis in both mucosal and submucosal layers, confirming the diagnosis of ischemic proctitis.

Patient had a slow recovery. The colostomy was reversed twelve months later with the fashioning of a colo-anal anastomosis.

DISCUSSION

Rectum has an extensive arterial supply network from the inferior mesenteric, internal iliac, internal pudendal arteries, and the marginal artery. This rich collateral supply explain why rectum is normally spared in ischemic colitis and hence the rare occurrence in literatures.

The exact mechanism for acute ischemic proctitis remains unknown. Physiological vasoconstriction secondary to mild central hypovolaemia has been shown to reduce intestinal mucosal perfusion, which might explain in part to the occurrence of acute ischemic proctitis[1,6]. This similar view was also expressed by Bharucha *et al*[1]. The hypovolaemic event in our patient would have therefore further compromised the arterial supply of the rectum, leading to the worsening of the ischemic proctitis.

Clinical presentation of patients with ischemic proctitis may be non-specific and often misleading, since they often present with lower abdominal pain, diarrhoea with bloody discharge, proctalgia, and rectal mucosal erosion on inspection. These presentations are also commonly seen in patients with inflammatory bowel disease, pseudomembranous colitis or neoplasia, and the distinction between these pathological entities can be difficult[7-9]. Histology is often required to confirm the diagnosis.

Conservative management is normally sufficient for majority of the cases of lower gastrointestinal bleeding. When conservative management fails, the aim for the subsequent treatment is to identify the source of bleeding, and coagulate the bleeding point. Emergency arteriography has been shown to be an effective option to locate the bleeding point, with a reported of successful localisation of active bleeding site between 40%-77%[10]. Our treatment was similar to the two-stage embolization approach described by Waugh *et al*[11]. We employed the super-selective technique for the initial embolization, and then a second more proximal embolization when the first attempt failed. Because histological diagnosis of ischemic proctitis from the initial biopsy was not available prior to both arteriogram interventions, rectal ischemia was aggravated by the embolization procedure in our case.

In retrospect, this case presented a clinical dilemma with a visualised extravasation in arteriogram ready for embolization in one hand, but with a co-existing pathology of ischemic proctitis in the other. Nevertheless, arteriogram embolizations offered window opportunities for adequate fluid resuscitation and stabilisation. As with the opinions of Waugh *et al*, arteriogram embolization allows the conversion of an emergency case to a semi-elective case scenario[11]. Due to the ability to localise an active bleeding site, a hemicolectomy can be performed as comparing to a subtotal colectomy, which carried a significantly higher mortality (10% *vs* 22%-37%, respectively)[12-15].

In conclusion, ischemic proctitis is difficult to diagnose clinically because of its rarity and close resemblance in clinical presentation to other pathological conditions. It represents a difficult management problem when there are co-existing clinical scenarios of a massive haemorrhage secondary to a bleeding vessel and an underlying ischemia. Emergency trans-catheterisation embolization can be a beneficial diagnostic and therapeutic tool in the management of similar cases, by providing a window period for optimal resuscitation prior to definitive surgical intervention.

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CASE REPORT

Large mucinous biliary cystadenoma with "ovarian-like" stroma: A case report

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Abstract

Hepatobiliary cystadenomas are rare tumors that are difficult to diagnose preoperatively. They can reach large sizes that make them real intraoperative "surprises". A 63-year-old woman, presented with a symptomatic huge abdominal cystic mass, underwent complete resection of the mass with safety margins. Histopathological report revealed mucinous hepatic cystadenoma with "ovarian-like" stroma and areas of sclerohyalinization. The differential diagnosis of the large cystic tumors which occupy the right hemiabdomen must include the biliary cystadenoma; the complete resection of the tumor with safety margins avoids local recurrence, and therefore represents the optimal therapy because of the malignant potential of the disease. The postoperative follow-up includes abdominal ultrasound or CT scan and CA 19-9 measurement.

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Key words: Liver; Cystic tumour; Mucinous cystadenoma; Surgery

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INTRODUCTION

Biliary cystadenomas are rare epithelial tumors with obvious malignant potential (20% of cases), and are frequently multilocular, predominantly located in the right hepatic lobe. They grow slowly and frequently are discovered accidentally during the medical investigations/surgical interventions performed for other conditions. They are more frequently encountered in women (95%)[1]; the maximum incidence is over the age of 40[2].

Cystadenomas represent less than 5% of the hepatic cystic tumors of biliary origin[3-4], their prevalence being 20 times lower than that of the simple hepatic cyst[3]. Based principally on ultrasound and CT, it has been reported that the prevalence of the biliary cystadenomas is much lower, probably 1000 times lower than that of the simple hepatic cyst[2]. In the speciality literature, there have been reported under 100 cases of biliary cystadenomas[5]. We report herein a case of large hepatic mucinous cystadenoma with mesenchymal stroma and a review of this condition based on the study of the speciality literature.

CASE REPORT

A 63-year-old woman was urgently admitted to the Surgical Clinic with intense abdominal pain located predominantly in the right upper quadrant, which started 5 d before, and was associated with nausea and pressure sensation. Physical examination revealed enlarged abdomen with tenderness in the right hemiabdomen, and a smooth-surfaced large mass (30 cm × 30 cm), without lumbar contact, difficult to mobilize and dull to percussion; as well as an irreducible, painless umbilical hernia with dimensions 4 cm × 4 cm.

Abdominal ultrasound showed a cystic mass with dimensions 24 cm × 18 cm × 24 cm, thin walls, multiple echoes inside and multiple septations posteriorly which delineated other small cysts with dimensions of maximum 5 cm; but homogeneous structure of the liver. Laboratory findings were within normal ranges. Contrast-enhanced abdominal CT scan showed a large abdominopelvic cystic mass that compressed the left hepatic lobe in its superior region, extending caudally to the uterine body and anteriorly to the peritoneal layer (Figure 1); and a septum and a dense mass (5 cm × 3 cm) could be distinguished in the posterior region of the described mass (Figure 2).

Surgery was performed through a supra- and subumbilical incision, which discovered a large cystic mass (25 cm × 25 cm) belonged to the hepatic segments III-IV-V, with the gallbladder lying over the mass (Figures 3 and 4). A total of 2500 mL of brown, mucinous fluid was evacuated by puncturing the cystic mass; and a mass with increased consistency and irregular surface was palpated in the posterior region of the tumor (Figure 5). Retrograde cholecystectomy, complete resection of the tumor and umbilical hernia repair were performed.

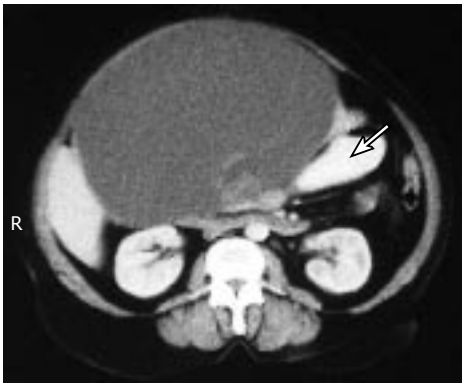


Figure 1 Contrast-enhanced CT scan demonstrates a large liver cyst in the left liver lobe (arrow).



Figure 2 CT scan shows the cyst with contrast-enhanced locules (arrow).



Figure 3 Intra-operative view of the large liver cyst.



Figure 4 Intra-operative aspects after puncture of the tumor. The gallbladder lies over the cyst's wall.

Cross-section of the specimen showed multiple septations in the inner surface and many locules with dimensions between 2-5 cm with mucinous content in the posterior region (Figure 6). Histopathological report revealed mucinous hepatic cystadenoma with "ovarian-like" stroma and areas of sclerohyalinization (Figure 7).

The postoperative course was uneventful ant the patient was discharge at the 10th postoperative day. A clinical check-up was carried out after a month, confirming the favorable evolution.



Figure 5 An area with increased consistency and irregular surface in the posterior region of the tumor.



Figure 6 The surgical specimen with multiple locules and septations.

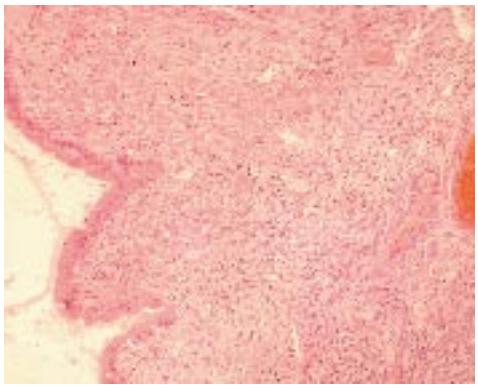


Figure 7 Photomicrograph demonstrates a columnar epithelium and an "ovarian-like" stroma (HE staining, × 200).

DISCUSSION

The problems that occur during the diagnosis of the large hepatic cystic tumors include the determination of the involved organs and differential diagnosis, especially with hepatic malignancies. Pathologically, two types of biliary cystadenomas are described: mucinous and serous[1]. Mucinous cystadenomas appear especially in women (95% of cases, possibly due to hormonal influence) and are located in intrahepatic region (84%), common bile duct (6%), hepatic ducts (4%), cystic duct (4%) and gallbladder

2%[1]. The pathogenesis of the tumor is unknown. It is considered that it has congenital origin from an aberrant intrahepatic biliary duct or from misplaced germ cells[2].

Biliary cystadenoma with mesenchymal stroma represents a tumoral histological variant that only affects women and has a columnar or cuboidal epithelium, an intermediary subepithelial layer of mesenchymal or fusiform cells, resembling ovarian stroma ("ovarian-like" stroma) and an external layer formed by a fibroconjunctive sheath that separates it from adjacent hepatic parenchyma, making a pseudocapsule. Biliary cystadenomas are usually multilocular .

Ultrasonography is the first paraclinical exploration used and shows an anechoic mass with hyperechoic internal septations and possibly mural papillary growths[4]. Abdominal CT demonstrates a single area with thin walls and internal septations; intravenous injection of contrast medium enhances the density of the cystic wall and may visualize the intracystic septations and the mural nodules[2,4]. MRI demonstrates the relationships of the tumor with local vascular structures and may bring informations about the nature of the intracystic fluid[6].Hepatic angiography shows an avascular lesion and possibly displacement of the intrahepatic vascular structures, but scintigraphy has a limited interest[2,4].Intra-operative ultrasound allows an exact identification of the site of the cystic mass and its relations with the adjacent biliovascular structures[7].

The serum CA 19-9 level may be elevated in biliary cystadenoma with mesenchymal stroma. It has been reported normalization of CA 19-9 level after the resection of the tumor, suggesting the possibility of using this investigation during the long-term post-operative follow-up of the patients[2]. The measurement of the CA 19-9 (marked increase but variable) and CEA (moderate increase) levels, originating from the intracystic fluid, differentiate these tumors from the simple and hydatid cysts of the liver, but cannot differentiate the biliary cystadenoma from cystadenocarcinoma[2]. Because of these causes, the differential diagnosis is difficult, especially with simple hepatic cyst, hepatic hydatid cyst and cystadenocarcinoma.

The evolution of biliary cystadenoma is slow with the possibility to occur various complications, out of which malignant transformation is of distinct importance. A study has demonstrated that 1/3 of hepatic cystadenocarcinomas arise from preexisting benign biliary cystadenomas[4].

The treatment of biliary cystadenoma is exclusively surgical. Complete resection of the tumor with intact hepatic resection margin is recommended, sometimes even hemihepatectomy[3]. This therapeutic approach is motivated by the malignant potential of the lesion and by the difficulty of the preoperative differential diagnosis with biliary cystadenocarcinoma. A study emphasizes that biliary cystadenoma with mesenchymal stroma may be enucleated because of the presence of a pseudocapsule which forms a dissection plane[4]. The indication for tumor enucleation is represented by small lesions, with central location, close to the hepatic hilum. Other performed operations, such as drainage through a Roux-en-Y loop, marsupialisation, percutaneous drainage followed by alcoholisation, partial cystectomy, and aspiration followed by partial excision, are associated with certain recurrence[2,5].

Postoperative follow-up is necessary; within the first year of operation, an ultrasound or CT scan of the liver should be performed after 6 mo, and then yearly. It is also useful to measure serum CA 19-9 level after the same time period.

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LETTERS TO THE EDITOR

Quantitative analysis using ELISA of vascular endothelial growth factor and basic fibroblast growth factor in human colorectal cancer, liver metastasis of colorectal cancer and hepatocellular carcinoma

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TO THE EDITOR

Angiogenesis consists of the sprouting of capillaries from pre-existing vessels[1]. It is well-known that tumor growth is angiogenesis-dependent. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) stimulated vascular endothelial cell proliferation and are involved in the neoplastic angiogenesis of several types of tumors including those of the intestinal tract[1-5]. Authors usually investigated VEGF and bFGF protein expressions using immunohistochemistry or Western blotting and VEGF and bFGF transcripts using reverse transcriptase polymerase chain reaction (RT-PCR). We recently reported in a previous issue of the *World Journal of Gastroenterology* that cirrhotic liver tissue levels of these two angiogenic growth factors can be easily evaluated by an enzyme-linked immunosorbent assay (ELISA)[6]. In order to confirm if this technical approach was suitable for tumors of the intestinal tract, we performed quantitative analysis of VEGF and bFGF in surgically resected tumors of colorectal cancer, liver metastases of colorectal cancer and hepatocellular carcinomas (HCC).

The procedure of the present study followed the rules edited by the French National Ethics. A total of 86 patients were investigated in the Department of Surgery of Limoges' CHU. Among them, 29 patients (18 men and 11 women, mean age 76 years) had a primary colorectal cancer, 30 patients (15 men and 15 women, mean age 64 years) had liver metastasis of colorectal cancer and 27 patients (26 men and 1 woman, mean age 62 years) had an HCC. Specimens for the tumor and the adjacent tissue were obtained during the surgical procedure and frozen at -80℃ until used. Tissue samples were homogenized in potassium phosphate buffer and VEGF and bFGF contents were detected using specific ELISA assays (DuoSet®, R&D Systems, Europe) according to the manufacturer's recommendations. Results in picogram per milligram (pg/mg) of wet weight are reported as mean ± SE. Differences in contents of VEGF and bFGF among tumor and the adjacent non-tumor tissue were analyzed by the Student *t*-test for paired data. A P value less than 0.05 was considered statistically significant.

As shown in Figure 1 (upper panel), VEGF contents were significantly elevated ($P = 0.0001$) in colorectal tumor (43.1 ± 9.0 pg/mg, $n = 29$) than that in the adjacent non-tumor tissue (5.3 ± 0.8 pg/mg, $n = 29$). Similarly, VEGF levels were markedly elevated ($P = 0.0001$) in liver metastasis of colorectal cancer (72.7 ± 9.5 pg/mg, $n = 30$) compared to the control tissue (14.7 ± 1.5 pg/mg, $n = 30$). In contrast, no significant differences were observed in VEGF levels between HCC (19.0 ± 2.3 pg/mg, $n = 27$) and the adjacent non-tumor tissue (18.4 ± 3.4 pg/mg, $n = 27$) ($P = 0.43$). As shown in Figure 1 (lower panel), bFGF contents were significantly reduced ($P = 0.016$) in colorectal tumor (67.0 ± 6.0 pg/mg, $n = 29$) compared to the adjacent non-tumor tissue (85.0 ± 6.0 pg/mg, $n = 29$). Similarly, VEGF levels were obviously lower ($P = 0.0001$) in liver metastasis of colorectal cancer (21.4 ± 3.4 pg/mg, $n = 30$) than that in the control tissue (39.3 ± 3.0 pg/mg, $n = 30$). VEGF levels were also reduced ($P = 0.0009$) in HCC (35.4 ± 7.3 pg/mg, $n = 27$) as compared with the adjacent non-tumor tissue (63.0 ± 4.6 pg/mg, $n = 27$).

The present study highlights that the ELISA method is a valuable method for the quantitative analysis of bFGF and VEGF contents in tumors located in the intestinal tract. We observed that bFGF contents in colorectal tu-

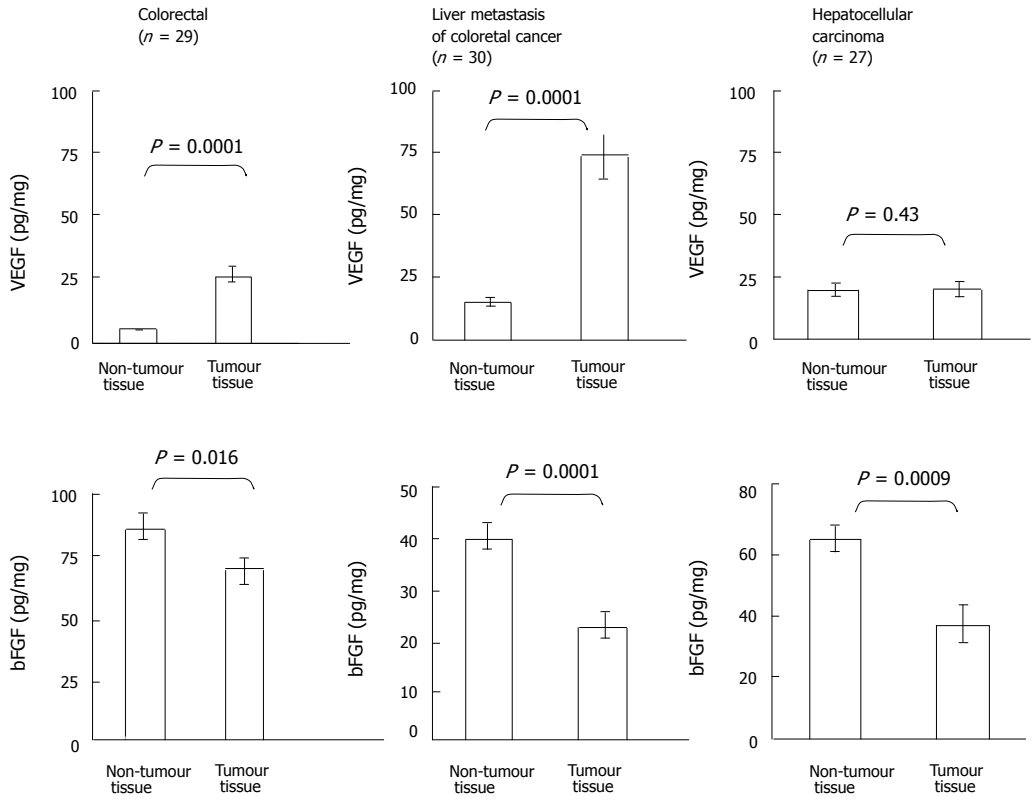


Figure 1 VEGF and bFGF contents in colorectal cancer, liver metastasis of colorectal cancer and HCC determined by ELISA. Specimens of tumors and adjacent non-tumor tissue were obtained during the surgical procedure. VEGF: Upper panel; bFGF: lower panel; *n*: number of patients. Results are expressed as mean ± SE.

mor, liver metastasis and HCC were significantly lower compared to the adjacent non-tumor tissues. This observation confirms a previous report investigating bFGF levels in human colorectal cancer[4]. At first sight, these results might be surprising since studies reported the involvement of bFGF in tumor growth. In fact, even though bFGF lacks a leader sequence for secretion, it is suggested that bFGF (localized to secretory granules) can be secreted by an alternative secretion pathway, accumulates in the extracellular matrix (ECM), from where it can be released by ECM-degrading enzymes. Our observation might, thus, be explained by secretion events inside tumors (leading to low bFGF tumor tissue content) and bFGF accumulation in tumor periphery (leading to high bFGF non-tumor tissue content). VEGF protein levels were markedly elevated in colorectal tumor and in their liver metastasis. These results are in agreement with the well known involvement of VEGF in colorectal tumor growth[7]. In contrast, VEGF protein levels were not elevated in HCC compared with surrounding non-tumor tissue. These results contrast with a previous study[2], but might be explain by the fact that a large number of our patients developed HCC in a cirrhotic liver; VEGF levels being elevated in cirrhotic tissue as compared with HCC ones[3,6].

In conclusion, results obtained with the ELISA procedure corroborate those obtained with other methods. It is, thus, tempting to speculate that the ELISA method would also be valuable for the quantitative analysis of other angiogenic growth factors and cytokines in tumors located in the intestinal tract.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
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cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
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7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
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Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
www.fascrs.org

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10th World Congress of the International Society for Diseases of the Esophagus
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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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EDITORIAL

Interaction or relationship between *Helicobacter pylori* and non-steroidal anti-inflammatory drugs in upper gastrointestinal diseases

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Abstract

According to a meta-analysis, *H pylori* and non-steroidal anti-inflammatory drugs (NSAID) independently and significantly increase the risk of gastroduodenal ulcer and ulcer bleeding. Their coincidence is frequent, demonstration of a possible relationship and consequent attitude is of important implications. But unfortunately, no consensus has been approved in the past years and their interactions are still controversial. *H pylori* and NSAID are known to share a number of pathogenic mechanisms, but there is no evidence for the significant synergic action between these two risk factors. Their relationship is independent, additive, synergistic or antagonistic without considering the influence of other factors because studies on this subject are different in almost all aspects of their methodology, including the definition of a NSAID user as well as the types, doses, duration and their indications for NSAID use, as well as their end-points, definition of dyspepsia and regimes used for eradication of *H pylori*. These might contribute to the conflicting results and opinions. *H pylori* infection in humans does not act synergistically with NSAID on ulcer healing, and there is no need to eradicate it. This notion is supported by the finding that the eradication of *H pylori* does not affect NSAID-induced gastropathy treated with omeprazole and that *H pylori* infection induces a strong cyclooxygenase-2 (COX-2) expression resulting in excessive biosynthesis of gastroprotective prostaglandin which in turn counteracts NSAID-induced gastropathy and heals the existing ulcer. Other investigators claimed that *H pylori* infection acts synergistically with NSAID on ulcer development, and *H pylori* should be eradicated, particularly at the start of long-term NSAID therapy. Eradication of *H pylori* prior to NSAID treatment does not appear to accelerate ulcer healing or to prevent recurrent ulcers in NSAID users. However, some recommendations can be drawn from the results of clinical trails.

H PYLORI AND ASPIRIN

Aspirin has a role in the prevention of cardiovascular and cerebrovascular disease, Alzheimer's dementia and several cancers. The widespread use of aspirin, however, is limited as many older subjects are currently unable to take aspirin because of gastrointestinal (GI) side-effects, such as gastroduodenal mucosal damage and increased risk of upper gastrointestinal bleeding. Cardiovascular patients on long-term low-dose aspirin have a stable risk of major upper GI bleeding, which is higher than that in controlled clinical trials[1]. Many aspirin users should receive prophylactic treatment since they often have several risk factors for upper GI complications. The best therapeutic approach for reducing GI toxicity in low-dose aspirin users is still ill-defined, because the causal relationship between intake of aspirin and *H pylori* appears to be more complex, but there is now wide agreement that *H pylori* infection increases mucosal damage and the risk of upper GI bleeding in low-dose aspirin users. In high-risk patients with a history of ulcer bleeding, curing *H pylori* infection alone without acid suppression has substantially reduced the risk of rebleeding caused by low-dose aspirin. Omeprazole appears to be very effective in reducing both acute gastroduodenal mucosal damage and upper GI bleeding in the high-risk patients taking low-dose aspirin, Thus, it appears that eradication of *H pylori* in patients who presented with GI bleeding and are scheduled to resume low-dose aspirin has the same efficacy as a daily dose of 20 mg of omeprazole for a 6-mo period. It was reported that it is not necessary to shift aspirin to clopidogrel in patients with GI bleeding[2], suggesting eradication of *H pylori* prevents recurrent peptic ulcer or ulcer bleeding in *H pylori*-positive patients, who continue to take low-dose-

aspirin. Maintenance therapy with proton pump inhibitor (PPI) is not generally required. However, it is reasonable to eradicate *H pylori* infection in those with increased risk of gastrointestinal complications (previous history of peptic ulcer, age over 65 years, concomitant use of corticosteroids, anticoagulants or individual non-steroidal anti-inflammatory drugs (NSAID) with higher risk for gastrointestinal complications, serious cardiovascular disease), but it does not guarantee complete protection and therefore a proton pump inhibitor should also be given. Switching from low-dose-aspirin to clopidogrel is not necessary. There are no valid data supporting *H pylori* eradication in low-risk patients on long-term therapy with low-dose-aspirin[3].

H PYLORI AND NSAIDS IN UNCOMPLICATED PUD PATIENTS

Whether *H pylori* eradiation plays a part in the healing and recurrence of ulcers in long term NSAID users remains unknown. It was reported that *H pylori* eradication does not confer any significant advantage on the healing of gastric and duodenal ulcers associated with long term NSAID use in arthritis patients[4], which is supported by a basic study[5] showing that *H pylori* infection is associated with increased cyclooxygenase-2 (COX-2) expression in gastric antral mucosa of both NSAID users and nonusers, but not in gastric ulcer, where the effect of NSAID inhibition plays a major role.

Further more, *H pylori* eradication is associated with significantly slower healing of gastric ulcers than in those not undergoing *H pylori* eradication treatment[6]. However, the effect of *H pylori* eradication on healing of NSAID induced duodenal ulcers does not appear to be so dramatic. There is evidence that it may be possible to prevent *H pylori*-associated duodenal ulcer by eradicating the infection. An evidence-based approach to treatment suggests that NSAID users should undergo *H pylori* eradication therapy if they have a duodenal ulcer, whether they continue NSAID. *H pylori* eradication should not be recommended universally or in high-risk gastric ulcer patients (female, older age and higher NSAID dose) who require management with acid suppression. Because there is evidence that *H pylori* infection does not potentate the risk of ulcer formation or ulcer complications in NSAID users. If such an effect occurs, it is likely to be relatively mild. Some data even indicate that[7] *H pylori* can protect against NSAID-induced gastric ulcers, suggesting that gastric ulcer healing is significantly enhanced in the presence of *H pylori* infection. Routine testing for and eradication of *H pylori* infection are not recommended for current takers of NSAID with no or low risk of peptic ulcers and its complications[8]. But more gastroenterologists believe that use of NSAID increases the risk of peptic ulcer in *H pylori* positive and negative patients and that *H pylori* eradication therapy should be given to all *H pylori*-infected patients with peptic ulcers irrespective of their use of NSAID[10].

Randomized trials comparing *H pylori* eradication to non-eradication in patients receiving NSAID showed that *H pylori* eradication reduces the incidence of peptic ulcer in the overall population receiving NSAID[4]. It appears to be especially effective in non-steroidal anti-inflammatory drug-naive patients. Nonetheless, *H pylori* eradication seems less effective than treatment with a maintenance proton pump inhibitor for preventing NSAID-associated ulcers.

The effect of *H pylori* eradication therapy on the healing of gastric ulcers remains controversial, but more gastroenterologists advise eradication of *H pylori* in this subgroup[9]. We recommend testing for and cure of *H pylori* infection in patients prior to the initiation of NSAID therapy and in those who are currently on NSAID and have a history of dyspepsia, peptic ulcer or ulcer complications. After eradication therapy, acid-suppressant therapy is advised to heal the ulcer. The success of *H pylori* eradication should always be confirmed because of the recurrent risk of peptic ulcer disease and bleeding in *H pylori*-infected patients[10].

The critical review of these studies indicates that the discrepancy arises mainly from heterogeneity in risk factors, patients and outcome measurements[11]. The main confounding factors are the type of treatment, nonaspirin NSAID or low-dose aspirin, different strains of *H pylori*, variable host response and type of ulcer. Therefore, both risks of *H pylori* and NSAID should be removed if possible, because it is not certain which factor is responsible for the ulcer[12].

H PYLORI AND NSAIDS USE IN COMPLICATED PEPTIC ULCERS

It was reported that the frequency of *H pylori* infection is significantly lower in patients with bleeding ulcers than in controls[13]. Interaction term, male multiple ulcers and NSAID use are independent risk factors for bleeding ulcers. There was a negative interaction between *H pylori* and NSAID use. Negative interaction between the two variables suggests that the presence of *H pylori* is associated with a lower risk of bleeding in ulcer patients taking NSAID. *H pylori* and NSAID use are independent risk factors for duodenal ulcer bleeding, whereas NSAID use is the main risk factor for bleeding gastric ulcers[14]. Interaction between these two factors is associated with reduced risk of bleeding gastric ulcers, but not of bleeding duodenal ulcers.

Established risk factors for NSAID-associated ulcer complications include advanced age, female gender, peptic ulcer history , use of non-selective NSAID and anticoagulant drugs or corticosteroids. Probable risk factors comprise *H pylori* infection and heavy consumption of alcohol, use of selective serotonin re-uptake inhibitors and smoking, *etc.* other factors. Knowledge of absolute risk estimates is important for clinical decision making[15].

Relapse of lesions in patients taking NSAID is highly site and type specific, and is not adversely affected by *H pylori* status, indicating that local mucosal factors predispose to ulceration in patients taking NSAID[16]. Identification of the responsible mucosal changes aids understanding and improves treatment. This finding

encourages analyzing the mechanism of relapse of peptic ulcer. After reviewing most of these articles we conclude that it *H pylori* eradication alone is insufficient in preventing recurrent peptic ulcer or ulcer bleeding in *H pylori*-positive patients on NSAID. Maintenance therapy with proton pump inhibitors (PPIs) or switching from nonselective NSAID to COX-2 inhibitors is required after eradication of *H pylori* in primary *H pylori*-negative patients with increased risk for gastrointestinal complications.

Clinically, maintenance treatment should be given after *H pylori* eradication if aspirin and or NSAID therapy is needed in patients with peptic ulcer bleeding. PPI significantly reduces the cumulative relapse of symptomatic and complicated ulcers in patients requiring NSAID after eradication of *H pylori*.

TREATMENT OF NSAIDS - RELATED DYSPEPSIA

NSAID-induced dyspepsia occurs in 10%-30% of patients treated with NSAID, leading to discontinuation of treatment in 5%-15%. Gastrointestinal tolerability of COX-2 selective inhibitors is better than THAT OF non-selective NSAID. *H pylori* infection does not influence gastrointestinal tolerability of NSAID. Therefore, patients should not be tested and treated for *H pylori* infection unless they have a history of peptic ulcer. Symptoms of NSAID-induced dyspepsia are poorly correlated with gastroduodenal mucosal damage. Therefore, upper gastrointestinal endoscopy should be performed only if symptom relief is not achieved with the first line empirical treatment and/or if symptoms suggestive of complications, such as bleeding. Proton pump inhibitors appear to be the treatment of choice for NSAID-induced dyspepsia[17].

H PYLORI AND COX-2

COX-2 inhibitors appear not to be ulcerogenic. The use of COX-2 inhibitors reduces significantly the gastrointestinal side effects of anti-inflammatory treatment. Management of *H pylori* in patients taking these drugs can be based upon the same risk assessment as in patients not taking NSAID. Treatment with celecoxib is as effective in patients with a recent history of ulcer bleeding as treatment with diclofenac plus omeprazole, with respect to the prevention of recurrent bleeding[18]. Selective COX-2 inhibitors (coxibs) can effectively treat pain and inflammation while reducing risk of gastropathy[19]. In case of patients with risk of NSAID-induced gastrointestinal toxicity, treatment with nonspecific NSAID should be selected[20].

SUGGESTIONS

Because the prevalence of *H pylori* infection and its associated diseases such as peptic ulcer and gastric cancer is different in different countries, it is not surprise to see different approvals for treatment. The clinical and cost-effectiveness advantage of *H pylori* screening have improved the patients' ulcer risk or the protective effect of *H pylori* eradication.

Many important issues should be considered when assessing the interaction between *H pylori* and NSAID. A better understanding of the interaction between *H pylori* and NSAID as well as the histological characteristics of *H pylori*-induced or NSAID-related gastropathy, contributes to the eradication of duodenal or gastric ulcer and their complications. Clinical trails should focus on strategies for subgroups of NSAID patients with *H pylori* infection. Multiple centers, big samples, double blind and randomized controlled clinical trails with comparable methodology should be performed to farther elucidate the interaction and relationship between *H pylori* and NSAID.

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REVIEW

Sphincter of Oddi dysfunction: Managing the patient with chronic biliary pain

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Abstract

Sphincter of Oddi dysfunction (SOD) is a syndrome of chronic biliary pain or recurrent pancreatitis due to functional obstruction of pancreaticobiliary flow at the level of the sphincter of Oddi. The Milwaukee classification stratifies patients according to their clinical picture based on elevated liver enzymes, dilated common bile duct and presence of abdominal pain. Type I patients have pain as well as abnormal liver enzymes and a dilated common bile duct. Type II SOD consists of pain and only one objective finding, and Type III consists of biliary pain only. This classification is useful to guide diagnosis and management of sphincter of Oddi dysfunction. The current gold standard for diagnosis is manometry to detect elevated sphincter pressure, which correlates with outcome to sphincterotomy. However, manometry is not widely available and is an invasive procedure with a risk of pancreatitis. Non-invasive testing methods, including fatty meal ultrasonography and scintigraphy, have shown limited correlation with manometric findings but may be useful in predicting outcome to sphincterotomy. Endoscopic injection of botulinum toxin appears to predict subsequent outcome to sphincterotomy, and could be useful in selection of patients for therapy, especially in the setting where manometry is unavailable.

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Key words: Sphincter of Oddi dysfunction; Manometry; Review

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INTRODUCTION

Sphincter of Oddi dysfunction (SOD) is characterized by symptoms of pancreaticobiliary obstruction in the absence of other structural causes. Symptoms attributable to SOD can be seen in three clinical scenarios: (1) the post-cholecystectomy syndrome; (2) acalculous biliary pain with an intact gallbladder; and (3) recurrent idiopathic pancreatitis. Its prevalence in the general population is 1.5%[1]. SOD is seen in 1% of patients after cholecystectomy, but in 14%-23% of patients with the post-cholecystectomy syndrome (biliary pain with elevated liver enzymes)[2,3]. This may reflect either disclosure of SO dysfunction after the reservoir function of the gallbladder is removed or that the symptoms leading to cholecystectomy are in fact caused by SO dysfunction. SOD can also be detected in 29% of patients with unexplained right upper quadrant pain and no evidence of gallstone disease[4], although due to symptom overlap many of these patients are not detected until after cholecystectomy. The prevalence of SOD in recurrent "idiopathic" pancreatitis ranges from 14.7%-72%[5-7]. A high index of suspicion is required to make the diagnosis after ruling out structural causes. Diagnosis is further limited by the wide spectrum of presentations (from predominantly structural SO stenosis to predominantly functional SO dyskinesia), as well as by the limited availability of invasive diagnostic techniques, such as manometry. We present herein a review of the literature and propose an approach to management of the SOD patient specifically in the setting where manometry is unavailable.

CLINICAL MANIFESTATIONS

Although SOD can be seen in any age group or gender, it is most commonly seen in women between the ages of 20 and 50 years[1]. Patients with predominantly biliary symptoms present with pain localized to the right upper quadrant or epigastrium lasting 30 min or more and should meet the Rome II diagnostic criteria for biliary-type pain[8]. To meet these criteria, the patients must have episodes of severe, steady pain in the epigastrium and right upper quadrant as well as all of: (1) episodes lasting 30 min or more; (2) symptoms occurring on one or more occasions in the previous 12 mo; (3) steady pain that interrupts daily activities or requires consultation with a

physician; and (4) no evidence of structural abnormalities to explain the symptoms. These patients may also have associated elevated conjugated bilirubin, transaminases or alkaline phosphatase, particularly during episodes of pain. A clinical classification system popularized by Geenen and Hogan separates patients with suspected biliary SOD into three groups based on the presence of objective signs of biliary obstruction[9]. Type I SOD is characterized by biliary pain as well as elevated liver enzymes (alkaline phosphatase and AST more than twice normal) on at least two separate occasions, a dilated common bile duct (> 12 mm at ERCP), and delayed contrast drainage from the bile duct (more than 45 min). Type II SOD is characterized by biliary pain and only one or two of the aforementioned objective criteria, and Type III SOD presents with only biliary pain and no objective criteria. This classification system has subsequently been modified by eliminating the delayed contrast drainage criterion as it is both cumbersome and often unreliable.

Type I patients are thought to have sphincter of Oddi stenosis as a cause for their symptoms and are likely to respond to endoscopic sphincterotomy. Type III patients represent a group of patients with true functional pain who may have sphincter of Oddi dyskinesia either alone or as part of a more diffuse smooth muscle dysmotility disorder. Type II patients represent an overlap between structural SO obstruction and a motility disorder, and the etiology can often be clarified by manometry. This clinical classification system also correlates with manometric findings, with elevated basal sphincter of Oddi pressures in 60%-85% of Type I, 18%-55% of Type II and 7%-28% of Type III patients[3,10].

When the pancreatic sphincter is involved, SOD can present with acute pancreatitis and should be considered in the differential diagnosis of recurrent idiopathic pancreatitis. A set of clinical criteria similar to those used to stratify biliary-type SOD has been utilized for pancreatic-type SOD[10]. Type I consists of recurrent pancreatitis and/or typical pancreatic pain and all of (1) elevated lipase or amylase more than one and a half times normal, (2) dilated pancreatic duct (head > 6 mm, body > 5 mm), and (3) prolonged pancreatic drainage time (> 9 min). Type II consists of typical pancreatic pain and only one or two of the other criteria, and Type III consists of typical pancreatic pain only. As in biliary pain patients, the clinical classification correlates with the likelihood of manometric abnormality with elevated basal pancreatic sphincter pressures in 92% of Type I, 58% of Type II and 35% of Type III patients[10].

PATHOGENESIS

The sphincter of Oddi is a smooth muscle sphincter which impedes biliary flow when contracted. It is composed of three sphincteric regions. First is the biliary sphincter, which is 10 mm long and controls biliary flow from the common bile duct. The pancreatic sphincter is slightly shorter (6 mm) and controls flow of secretions through the pancreatic duct. Finally, a 6-mm long common sphincter encircles the confluence of the pancreatic and bile ducts as they near the duodenal lumen[11]. A component of the sphincter is intraluminal, and it is this portion that is transected during endoscopic sphincterotomy. The etiology of SOD is not entirely clear. Pain is presumed to be due to obstructed biliary or pancreatic flow leading to increased upstream pressure. This is especially evident in the post-cholecystectomy population where removal of the gallbladder may eliminate a reservoir for backflow of bile. Elevated sphincter pressure has also been shown to produce acute pancreatitis in an animal model[12]. In some patients, there may be fixed obstruction at the level of the papilla. Biopsies of the ampullary region show inflammation or fibrosis in 43% of patients with SOD[13]. These patients may correspond to Type I SOD with a fixed "papillary stenosis" picture.

Microlithiasis has been proposed as a possible contributing factor by creating a transient obstruction. However, only 3.5%-5% of patients with symptoms of SOD have microlithiasis detected in bile samples[14,15]. In addition, there is no difference in the frequency of microlithiasis between symptomatic patients with elevated sphincter pressures and those with normal manometry[14,15], so microlithiasis is not likely to explain symptoms in these patients. Another potential cause for SOD symptoms is a motor abnormality of the sphincter of Oddi, or "biliary dyskinesia". In addition to elevated resting basal sphincter pressures, SOD has been associated with increased frequency of phasic contractions, increased retrograde propagation of contractions and a paradoxical response to cholecystokinin (CCK)[16]. A paradoxical response to CCK (failure to completely obliterate phasic contractions) is seen more frequently in SOD patients with concomitant irritable bowel syndrome, indicating the possibility of a global gut motility disorder[17]. Supporting this theory is the presence of increased phase II and III migrating motor complex activity in the small bowel of post-cholecystectomy patients with objective evidence of SOD[18].

SOD patients also exhibit increased non-gastrointestinal symptomatic complaints and increased rates of childhood sexual abuse compared to controls[19], similar to that seen in irritable bowel syndrome. In one series, a formal diagnosis of somatization disorder was made in 30% of women with SOD and none of controls. In patients with Type III SOD (those with no objective signs of biliary obstruction), increased rates of depression, obsessive compulsive traits and anxiety compared to controls are also seen[20]. Visceral hyperalgesia is postulated to cause pain in irritable bowel syndrome, and indeed duodenal hyperalgesia as assessed with duodenal barostat was confirmed in SOD Type III when compared to controls[20]. Interestingly, no evidence of rectal hyperalgesia was detected in Type III SOD patients compared to controls[20], while irritable bowel patients had significantly lower rectal pain thresholds compared to SOD patients and controls[21]. Thus, at least in Type III SOD, visceral hyperalgesia and psychiatric factors may play a role in symptoms, necessitating a multidisciplinary approach to pain control.

DIAGNOSIS

Diagnostic evaluation in patients with pancreaticobiliary

symptoms should begin by ruling out structural causes for pain. Liver biochemistry as well as pancreatic enzymes should be drawn. Radiologic imaging is somewhat site-dependent but may include transabdominal ultrasound, endoscopic ultrasound, CT scan, MRCP, ERCP and analysis of bile for crystals. Up to 4.3% of patients with suspected SOD may have an ampullary tumor as the cause of their symptoms, so biopsies of the ampullary region should be considered[13]. When no other explanation for a patient's symptoms can be found, a variety of diagnostic methods exist to confirm the diagnosis of SOD.

Manometry

Manometry is the accepted gold standard for diagnosis of SOD since symptoms are thought to stem from obstructed biliary or pancreatic flow at the level of the sphincter of Oddi. Manometry findings have been shown to be reproducible[22] with good interobserver variability[23]. Geenen *et al*[24] found manometric findings were stable over time when repeated. However, these results were recently refuted in a group of patients with normal manometry and persistent symptoms[25]. Repeat manometry in this group of 12 patients diagnosed SOD in five patients with eventual symptom relief after endoscopic therapy. In the absence of a pathologic gold standard for comparison, most studies have used elevated basal sphincter pressure when compared to duodenal pressure as diagnostic of SOD[24,26]. An elevated basal sphincter pressure is defined as > 40 mmHg (3 SD greater than that of healthy volunteers)[27]. This SO hypertension is deemed likely to correlate with SO obstruction, and indeed most studies have found a correlation between SO hypertension on manometry and outcome after endoscopic sphincteroto my[24,28-30]. Other motility abnormalities include increased frequency of phasic contractions (> 8/min), increased retrograde propagation of phasic contractions (> 50%), and paradoxical response to cholecystokinin[16], although these findings of "SO dysmotility" have generally not been used to select patients in outcome studies. In fact, the one study that did compare outcome after sphincterotomy in patients with SO hypertension and those with other markers of dysmotility found no difference in outcome between patients with normal manometry and those with SO dysmotility, whereas patients with elevated basal sphincter pressures had a significant benefit after sphincterotomy[31]. Thus, although sphincter dysmotility may be seen in patients with SOD symptoms, it is not successfully treated with sphincterotomy, possibly because it causes pain through mechanisms other than obstruction to biliary flow.

When SO manometry is performed for presumed SOD, it is now generally accepted practice to measure pressures at both the biliary and pancreatic sphincter. Discordant results are obtained from the pancreatic and biliary sphincters in 30%-48% of patients[6,32,33], indicating that manometry should be performed at both sphincters so as not to misdiagnose patients who could ultimately benefit from therapy. Manometry is limited by its availability and the level of technical expertise required for its performance. One must be cautious that the drugs used for sedation do not alter SO motility, as midazolam has been found to reduce basal SO pressure[34] and narcotics to cause SO spasm[35]. Diazepam does not appear to have an effect on SO motility and may be safely used as sedation in this setting[36]. Manometry is also an invasive diagnostic procedure with a potential risk of pancreatitis in 17%-27% of cases[37,38]. Much of the risk of pancreatitis seen after manometry appears to be attributable to concomitant ERCP as a significantly lower rate of pancreatitis is seen in patients who undergo manometry alone compared to manometry with ERCP (9.3% *vs* 26.1%)[37], and there is no significant difference in pancreatitis rates between SOD patients undergoing ERCP alone compared to ERCP plus manometry (*OR* = 0.72)[38]. However, THE 9% pancreatitis risk seen in patients who underwent SO manometry alone is still a significant adverse outcome that must be weighed against the benefits of this diagnostic modality.

Finally, although manometry has been shown to correlate with endoscopic outcome in many studies as previously mentioned, it is not 100% sensitive. In a study by Rolny *et al*[39], 35% of Type I SOD patients had normal manometry but still benefited from endoscopic sphincterotomy. Similarly, in a study by Wehrmann *et al*[40], manometry findings were similar between Type II and Type III SOD patients, but therapeutic response was significantly better in patients with Type II SOD. Because of the imperfect predictive value of manometry as well as its limited availability and potential for adverse effects, much attention has been paid to non-invasive diagnostic techniques.

Quantitative hepatobiliary scintigraphy

Hepatobiliary scintigraphy (HBS) is performed in the fasting state and involves administration of a radionuclide tracer to quantitate biliary flow. In the setting of SOD, obstructed biliary flow is expected which can be quantitated using a number of outcome measures. Intravenous CCK infusion is used to enhance bile flow and aid in the demonstration of obstruction. Although an attractive alternative to manometry due to its non-invasive nature, studies of HBS have been limited by the lack of a standardized protocol and the use of a variety of outcome measures [duodenal appearance time (DAT), hilum-to-duodenum transit time (HDTT), or a composite scintigraphic score]. The variability in study protocol may in part explain the wide range of reported sensitivities (from 25 to 100%) and specificities (from 78% to 100%)[41-43].

An early study by Sostre *et al*[43] reported a sensitivity and specificity of 100% for the diagnosis of SOD using a scintigraphic score composed of (1) time to peak liver activity, (2) time of first visualization of the intrahepatic biliary tree, (3) biliary tree prominence, (4) time of visualization of bowel, (5) percent common bile duct emptying, and (6) CBD to liver ratio. However, these results were from a small series of twelve patients, and only seven of these patients had elevated basal sphincter pressure. These impressive results have not been replicated to date.

Using HDTT, DAT and half time of excretion as outcomes, Madacsy *et al*[44] found a significant difference in scintigraphic parameters between SOD patients with elevated sphincter pressures and asymptomatic controls.

They found HDTT to be the most sensitive measure with a sensitivity of 89%. However, this was a small study with twenty controls and twenty patients, of whom only nine had elevated basal sphincter pressures. Subsequently, a study of 29 patients with persistent post-cholecystectomy pain undergoing both manometry and scintigraphy showed a sensitivity of only 25%-38% and specificity of 86%-89% when using various cut-off values for the previously described scintigraphic score[41]. The sensitivity of HDTT was equally poor at 13%, although the specificity was 95%. No correlation was found between basal SO pressure and HDTT value. Similarly, a prospective series of 304 patients with suspected SOD compared HBS to manometry as the gold standard. Sensitivity and specificity were 49% and 78%, respectively, using hepatic peak, half time and percent CBD clearance as outcome measures[42]. These larger, prospective studies using manometry as a gold standard showed that HBS is not sensitive or specific compared to manometry as a diagnostic modality.

Interobserver variability and reproducibility of the scintigraphic score was examined in a group of asymptomatic post-cholecystectomy patients with normal liver enzymes[45]. Interobserver agreement was good (κ = 0.507), but when two scans for each patient were compared there was poor agreement (κ = 0.062), indicating poor reproducibility. Interestingly, in this asymptomatic population, 40% had at least one of their two HBS studies reported as abnormal, indicating a significantly lower specificity than previously reported for this score. Although correlation between HBS and manometry seems to be poor, one must remember that manometry is not a perfect gold standard. The most important outcome to be considered is response to therapy, and it is possible that a functional test of bile flow may detect patients with normal manometry who may nonetheless respond to endoscopic sphincterotomy. This was demonstrated by Cicala *et al*[46] in a group of Type I and Type II SOD patients where prolonged HDTT predicted a response to endoscopic therapy in 93% of cases, as compared to only 57% of responses predicted by manometry. In this study, all Type I patients had abnormal scintigraphy studies, as did 64% of Type II patients, and all patients with abnormal manometry also had abnormal HBS. In a different study which showed a poor correlation between HBS and manometry, HBS predicted 86% of patients who subsequently had a long-term symptomatic response to sphincterotomy[42]. Thus, although the role of HBS in diagnosing SOD remains undetermined, it may have a place in predicting therapeutic outcomes and determining which patients should undergo endoscopic sphincterotomy. Variations on the HBS technique may also improve its diagnostic ability. Morphine provocation to accentuate sphincter of Oddi spasm during HBS produced a significantly different percent excretion of tracer between patients with normal and abnormal SO motility[47]. Sensitivity was 83% and specificity 81% for this technique compared to manometry as a gold standard. A functional obstruction in SOD patients was demonstrated by the administration of glyceryl trinitrate which reversed the abnormal HBS findings in a group of patients with abnormal SO manometry, while having no effect on patients with mechanical biliary obstruction[48]. It is possible that further refinement of these techniques may aid in diagnosis of SOD and prediction of outcome to therapy, but further trials with response to sphincterotomy as an outcome are needed.

Fatty meal ultrasonography

Fatty meal ultrasonography (FMU) is performed by ingestion of a standardized fatty meal with ultrasound performed before and 45 min after ingestion. The fatty meal stimulates release of CCK which induces bile flow. In the setting of a functional obstruction to bile flow, this causes dilation of the diameter of the common bile duct when compared to baseline. Sensitivity of FMU when compared to manometry in SOD patients is only 21%, although specificity is 97%[42]. Similar to HBS, however, 87% of patients with abnormal FMU results did have a therapeutic response to sphincterotomy, indicating a possible role in selection of patients for therapy. The utility of both FMU and HBS for diagnosis is further limited because their accuracy falls as objective signs of SOD decrease. Ninety percent of SOD Type I patients had abnormal findings in at least one of these non-invasive tests, as compared to only 50% with Type II and 44% with Type III SOD[42]. Type I patients have more objective signs of biliary obstruction and pose less of a diagnostic dilemma than Type II and III patients who are the patients who would most benefit from a non-invasive diagnostic test prior to contemplating invasive therapy such as sphincterotomy. This limitation further restricts the use of non-invasive diagnostic testing at this time.

Non-invasive testing of the pancreatic sphincter

Fatty meal ultrasonography and hepatobiliary scintigraphy assess bile flow, but SOD can also present with pancreatitis due to a hypertensive pancreatic sphincter. Non-invasive testing of the pancreatic portion of the sphincter of Oddi has centered on the use of secretin to stimulate pancreatic flow with subsequent assessment of pancreatic ductal dilatation as a marker of obstructed flow. The secretin test was initially performed with trans-abdominal ultrasound, and was found to have a sensitivity of 88% and a specificity of 82% when compared to manometry in patients with recurrent idiopathic pancreatitis[49]. Not surprisingly, concordance with results from pancreatic manometry was superior to concordance with manometry findings from the biliary sphincter. An endoscopic ultrasound-guided secretin test has also been described[50]. A lower sensitivity of only 57% and specificity of 92% were reported with this technique, although only 20 patients with SOD were examined. Again, the secretin test was less accurate in detecting a hypertensive biliary sphincter as compared to pancreatic sphincter hypertension. A preliminary study of secretin MRCP in 15 patients with recurrent idiopathic pancreatitis showed concordance in 13 (87%) of these patients[51]. The two patients in whom MRCP results were false negatives were patients with normal basal sphincter pressures but some other form of sphincter dysmotility. These results are intriguing, and MRCP could have the added benefit of ruling out structural causes of pancreaticobiliary symptoms in these patients, reducing the need for invasive testing, such as ERCP and SO

manometry, in this high-risk group of patients. Further evidence of the utility of secretin MRCP is necessary at this time.

THERAPY

Medical therapy

Medical therapy for SOD has been disappointing. Calcium channel blockers, such as nicardipine, have been shown to decrease sphincter of Oddi pressure in healthy subjects[52]. In small placebo-controlled, crossover studies, nifedipine also decreased biliary-type pain in patients with Type II sphincter of Oddi dysfunction[53,54], however, these trials were of short duration (twelve to sixteen weeks of active treatment). A more recent attempt at a longer duration of nifedipine therapy for sphincter of Oddi dysfunction (types II and III) was terminated due to unacceptably high rates of side effects, including peripheral edema, headache and dizziness[55] and did not show a reduction in pain episodes compared to placebo. The effect of nitrates on the sphincter of Oddi has also been investigated. Oral nitrates have been shown to reduce sphincter of Oddi pressure[56,57] but no clinical trials have assessed their use in SOD, and therapy is likely to be limited by side effects, such as headache. Overall, medical therapy to date has been limited by medication side effects, the inconvenience of long-term oral therapy, and the possibility of tachyphylaxis, and is not currently standard of care for sphincter of Oddi dysfunction. Other avenues of potential medical treatment remain to be explored. Intravenous octreotide effectively decreases basal and peak sphincter pressures in SOD patients when compared to a placebo infusion[58]. Clinical trials to determine whether this effect translates into improved clinical endpoints would be beneficial, especially with the availability of a long-acting injectable form of octreotide. Preliminary data with the prostaglandin E1 analogue, alprostadil alfadex, as well as the protease inhibitor, gabexate mesilate showed relaxant effects on the sphincter of Oddi[59,60], and their clinical utility also remains to be explored. One small study showed a relaxant effect on sphincter of Oddi motility with the use of electroacupuncture at an acupoint thought to be related to the hepatobiliary system when compared to a control acupoint[61]. A reduction in basal sphincter pressure as well as phasic contraction was seen during electroacupuncture, which was associated with elevation of plasma CCK levels. It remains to be seen whether this novel therapy could present a non-invasive therapeutic option for SOD.

Endoscopic therapy

Since the clinical symptoms of sphincter of Oddi dysfunction are thought to stem from a functional obstruction at the level of the sphincter, sphincterotomy to alleviate this obstruction would seem a promising therapeutic avenue. This is especially true in Type I SOD, where objective features of biliary obstruction (elevated liver enzymes and a dilated common bile duct) are present. Indeed, Rolny *et al*[39] treated a group of seventeen Type I SOD patients with sphincterotomy and found that despite the fact that sphincter of Oddi hypertension was not found in 35% of patients at manometry, all patients experienced pain relief after sphincterotomy for a mean follow-up of 28 mo (Table 1). These results were replicated in a series reported by Sugawa *et al*[62], in which Type I SOD patients underwent sphincterotomy without prior manometry. All patients in this series experienced symptom resolution at 26 mo of follow-up[62]. Based on these outcomes, sphincterotomy is recommended in Type I patients without prior manometry, since these patients are likely to have a clinical improvement regardless of manometric findings.

Type II and III patients have fewer clinical manifestations of high grade biliary obstruction, and correspondingly are less likely to respond to endoscopic sphincterotomy (Table 1). In a landmark trial by Geenen *et al*[24], 47 Type II SOD patients were randomized to sphincterotomy or sham. Manometry was also performed. For patients with elevated sphincter of Oddi pressure, 10 of 11 patients who underwent sphincterotomy had improvement in pain scores at one year, as compared to only 3 of 12 patients who underwent sham. This benefit was also seen when follow-up was extended to four years, with a 94% clinical response rate after sphincterotomy in patients with elevated basal sphincter pressures as compared to clinical improvement in one third of patients with normal baseline pressures regardless of treatment. Since patients with normal manometry had no difference in pain relief between the sphincterotomy and sham groups, SO manometry is recommended in Type II SOD patients prior to subjecting them to the possible adverse effects of a sphincterotomy. Subsequent studies have both confirmed the efficacy of sphincterotomy for patients with elevated basal pressures and shown no response in patients with normal pressures or with sphincter dyskinesia[28-31]. Sphincterotomy is then recommended only in Type II patients with elevated basal sphincter pressures.

Subsequent studies of endoscopic therapy have included patients with Type III SOD, who have no objective findings of biliary obstruction. A retrospective analysis showed no difference in baseline sphincter hypertension between Type II and Type III patients (60% *vs* 55%, respectively), and subsequently showed no difference in the proportion of Type II and Type III patients in response to sphincterotomy (68% *vs* 56%, respectively)[63]. A prospective trial by Wehrmann *et al*[40] confirmed that a similar proportion of Type II and Type III patients had manometric findings of SOD, but found that only 8% of Type III patients had a sustained clinical improvement at 2.5 years of follow-up, as compared to 60% of Type II patients. Based on these findings, manometry in Type III SOD patients should be approached with caution as it both carries procedural risks and does not reliably predict outcome to sphincterotomy.

Risks of sphincterotomy

Sphincter of Oddi manometry is limited by its availability as well as potential complications of the procedure. Indeed, SOD has been shown to carry an increased risk of post-ERCP pancreatitis with frequencies ranging from 12.5% to 27%[58,64,65] as compared to 3%-5% of patients who undergo ERCP for bile duct stones. In patients with

Table 1 Sphincter of Oddi dysfunction: Outcome after sphincterotomy

Author, yr	*n*	Milwaukee Class	Response rate (%)	Duration (mo)	Pancreatitis (%)
Neoptolemos[29] 1988	30	NS	19/30 (63%) Manometry in 23 patients: Man + ve 15/18 (83%) Man – ve 0/5 (0%)	46	4/30 (13%)
Rolny[39] 1993	17	Type Ⅰ	17/17 (100%)	28	NS[1]
Sugawa[62] 2001	8	Type Ⅰ	8/8 (100%)	26	0
Geenen[24] 1989	23 sphincterotomy (24 sham Rx)	Type Ⅱ	15/23 (65%) Man + ve[2] 10/11 (91%) Man - ve[2] 5/12 (42%)	12	2/47 (4%)
Toouli[31] 2000	37 sphincterotomy (42 sham Rx) (2 excluded)	Type Ⅰ (9) Type Ⅱ (72)	11/13 (85%) man+ve[2] 12/24 (50%) man-ve[2]	24	7/81 (9%)
Fullarton[28] 1992	10	Type Ⅱ	8/10 (80%) all man+ve[2]	24	NS[1]
Lin[72] 1998	24	Type Ⅱ	19/24 (79%) Enzyme+ve 18/20 (90%) Enzyme-ve 1/4 (25%)	18	2/24 (8%)
Botoman[63] 1994	43	Type Ⅱ (21) Type Ⅲ (22) man+ve	Type Ⅱ 14/21 (67%) Type Ⅲ 12/22 (55%)	37	Type Ⅱ 16% Type Ⅲ 15%
Bozkurt[30] 1996	23	Type Ⅱ and Ⅲ	19/23 (83%) man+ve[2]	8-62	NS[1]
Wehrmann[40] 1996	37 (with + ve manometry)	Type Ⅱ (22) Type Ⅲ (15)	Type Ⅱ 13/22 (60%) Type Ⅲ 1/15 (8%)	30	NS[1]

[1]NS= not specified; [2]Man+ve = manometry positive; Man-ve = manometry negative.

SOD, manometry does not appear to independently increase the risk of pancreatitis, but pancreatitis is increased with biliary sphincterotomy (*OR* = 5.13) and pancreatography (*OR* = 11.32)[38]. Since pancreatitis in this setting is thought to result from edema at the level of the sphincter of Oddi, trials of pancreatic stents to improve pancreatic drainage have been performed. Although an early study did not show a significant benefit for pancreatic stenting[66], subsequent trials have shown a significant reduction in pancreatitis in patients who have a pancreatic stent placed at the time of biliary sphincterotomy[67-69]. A subsequent meta-analysis confirms the benefit of pancreatic stenting to decrease pancreatitis in high-risk patients (including sphincter of Oddi dysfunction) with an odds ratio of 0.3 and a number needed to treat of eleven[70]. Preliminary evidence with botulinum toxin injection into the pancreatic sphincter to prevent pancreatitis after biliary sphincterotomy shows a trend towards benefit. Six of fourteen (43%) patients in the sham arm developed pancreatitis compared to three of twelve (25%) patients in the botulinum toxin arm[71]. Until more trials with botulinum toxin become available, the use of prophylactic pancreatic duct stenting in these high risk patients should be strongly considered, especially if additional risk factors such as a small common bile duct are present.

Selecting patients for sphincterotomy

Because of the problems of availability and adverse effects, non-invasive surrogate markers predicting outcome after sphincterotomy have been sought with conflicting results. Retrospective analysis has shown a better outcome after sphincterotomy in patients with greater common bile duct diameter or delayed contrast drainage from the bile duct[29]. Another retrospective series of Type Ⅱ SOD patients did not find common bile duct diameter to be predictive of outcome after sphincterotomy, but did find elevated liver enzymes to predict clinical resolution after endoscopic therapy[72]. Patients in this series did not have SOD confirmed by manometry, and some of the response after sphincterotomy may have been due to microlithiasis, which would also have caused elevated liver enzymes. However, in prospective studies, none of elevated liver enzymes, common bile duct diameter, common bile duct drainage or a morphine-prostigmine test predicted outcome after sphincterotomy[24,31].

Quantitative choledochoscintigraphy has been compared to manometry as a predictor of outcome after sphincterotomy. Scintigraphy has the advantage of providing functional information regarding bile flow, as opposed to the structural information provided by manometry. Scintigraphy showed a prolonged hepatic hilum-duodenum transit time (HHDT) in all eight Type I SOD patients studied as well as 64% of Type Ⅱ SOD patients. HHDT was abnormal in all of the patients with abnormal manometry as well as 46.66% (7/15) patients with normal SO pressure[46]. In 14 patients who subsequently underwent sphincterotomy, symptomatic improvement was accurately predicted by scintigraphy in 13 (93%), while manometry predicted improvement in only eight (57%). Scintigraphy is thus a promising non-invasive method for prediction of outcome after sphincterotomy, even in patients with normal manometry who may have a functional obstruction to

bile flow causing their biliary-type pain. Larger studies are needed to confirm these findings.

Biliary stenting may predict outcome after sphincter ablation. In a small prospective series, 90% of patients who had a symptomatic response 12-14 wk after placement of a biliary stent subsequently responded to sphincterotomy[73]. The stent trial had a positive result in all of the 7 patients with elevated sphincter of Oddi pressure (6 of whom later responded to sphincterotomy) as well as in 16 patients with normal manometry (of whom 5 subsequently responded to sphincterotomy). Although a biliary stent trial may detect some patients with normal manometry who may subsequently benefit from sphincter ablation, it requires a second invasive procedure to be performed in a significant proportion of patients who do not benefit, and thus is not a viable option to select patients for sphincterotomy. In the absence of reproducible non-invasive predictors of response to sphincterotomy, some have suggested performing empirical sphincterotomy in Type II SOD without prior manometry[74]. Manometry-guided therapy was compared to empiric sphincterotomy with a decision analysis model, and the empiric sphincterotomy pathway was shown to cut costs from $2790 per patient to $2244 per patient. Fifty-five percent of the manometry-guided group was predicted to experience symptom resolution, as compared to 60% in the empiric sphincterotomy group. Randomized, controlled trials to compare these two treatment options are necessary, but in centres where manometry is unavailable, empiric sphincterotomy may be justifiable.

Botulinum toxin

Botulinum toxin acts by inhibiting presynaptic release of acetylcholine to inhibit contraction in smooth and striated muscle. Because of this property, it has been used for a variety of gastrointestinal disorders characterized by elevated muscle tone, including achalasia, gastroparesis and anal fissure[75]. Botulinum toxin has been shown to reduce sphincter of Oddi pressure in animals[76], and subsequently in case reports in patients with SOD[77,78]. Two uncontrolled case series have examined the clinical effect of botulinum toxin for both biliary type SOD[79] and for recurrent pancreatitis due to pancreatic SOD[80]. In patients with biliary type pain (type III SOD), endoscopic sphincterotomy is of uncertain benefit[40], and given the high risk of pancreatitis[64] in patients with SOD, a less invasive option such as botulinum toxin injection is attractive. Of 22 type III SOD patients treated with 100-unit botulinum toxin injection, 12 had a resolution of symptoms[79]. Eleven of these patients underwent endoscopic sphincterotomy when they developed recurrent pain, and all of these patients responded to sphincterotomy. In contrast, none of the botulinum toxin non-responders experienced symptom resolution after sphincterotomy despite a significant reduction in sphincter of Oddi pressure, indicating that a hypertensive sphincter of Oddi was not the cause of their abdominal pain. A trial of botox injection had a positive predictive value of 100% and a negative predictive value of 80% in predicting the outcome of sphincterotomy, illustrating the potential utility of using botulinum toxin injection as a diagnostic and

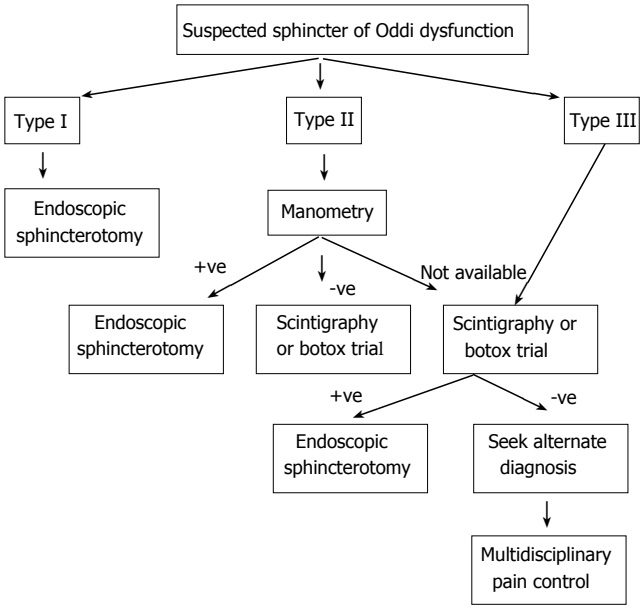


Figure 1 Sphincter of Oddi Dysfunction: Management algorithm.

therapeutic trial before proceeding to the more invasive procedure of sphincterotomy. Similarly, in patients with recurrent pancreatitis and elevated pancreatic sphincter of Oddi pressures, 80% (12/15) patients had no further pancreatitis attacks for a mean of six months after botulinum toxin injection[80]. These patients then experienced long-term remission after sphincterotomy.

In both of these series, botulinum toxin was effective, free of significant side effects and was relatively easy to administer, as cannulation of the papilla was not required. Unfortunately, its role as a therapy is limited by its duration of efficacy. Muscular function usually returns within two to six months after injection[75]. If these results can be replicated and extended at other centers, botulinum toxin can act as a diagnostic trial when manometry is unavailable to predict which patients would subsequently benefit from sphincterotomy.

In conclusion, sphincter of Oddi dysfunction is a disorder that should be suspected in patients with chronic biliary pain or recurrent pancreatitis when other organic causes have been ruled out. Because of the lack of a pathologic gold standard, manometry has been adopted as the *de facto* standard diagnostic test despite the fact that it does not have perfect correlation with outcome to therapy, it is invasive and it is available only at specialized centers. In patients with Type I SOD, a good response to sphincterotomy can be predicted regardless of manometric findings, and these patients should proceed directly to sphincterotomy (Figure 1). Type II SOD patients are the group who can most benefit from manometry to select patients for sphincterotomy. If manometry is not available, hepatobiliary scintigraphy or possibly a botulinum toxin trial can be used instead to predict response to sphincterotomy. Finally, Type III SOD patients pose a particular therapeutic challenge and likely represent a chronic pain disorder or dysmotility syndrome. The risks of manometry in this group outweigh the benefits and here scintigraphic studies

or a botulinum toxin trial may also be effective in predicting response to sphincterotomy. Further research is needed in many areas regarding sphincter of Oddi dysfunction. Larger trials of non-invasive diagnostic modalities are needed, with therapeutic outcome as an endpoint. Clinical trials comparing outcomes to sphincterotomy after selection with manometry compared to empiric sphincterotomy would guide therapy in Type II patients. Finally, there is little information regarding treatment of patients who have an incomplete response to sphincterotomy. Answering these questions will bring us closer to a more evidence-based mode of managing these patients.

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Tumor differentiation phenotype in gastric differentiated-type tumors and its relation to tumor invasion and genetic alterations

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Abstract

AIM: To clarify the relations between tumor differentiation phenotype and tumor invasion or genetic alterations in gastric differentiated-type tumors.

METHODS: We examined the tumor differentiation phenotype, the presence of mutations in *APC* and *p53*, and the microsatellite instability (MSI) status in 48 gastric adenomas and 171 differentiated-type carcinomas. The tumor differentiation phenotype was determined by examining the expression of human gastric mucin (HGM), MUC6, MUC2 and CD10. The tumors were then classified into gastric- (G-), gastric and intestinal mixed- (GI-), or intestinal- (I-) phenotypes, according to the immunopositivity of the above markers. The presence of mutations in *APC* and *p53* and the MSI status were also investigated in all the tumors.

RESULTS: Gastric adenomas were significantly associated with CD10 expression, I-phenotype tumors and the presence of *APC* mutations, compared with carcinomas (66.7% *vs* 25.1%, $P < 0.0001$; 56.3% *vs* 14.6%, $P < 0.0001$; 39.6% *vs* 14.0%, $P < 0.0001$, respectively) and inversely associated with expressions of HGM and MUC6 and the presence of *p53* mutations (10.4% *vs* 62.6%, $P < 0.0001$; 39.6% *vs* 64.3%, $P = 0.003$; 2.0% *vs* 26.3%, $P = 0.001$, respectively). The frequency of *APC* mutations was significantly higher in HGM-negative tumors, MUC6-negative tumors, CD10-positive tumors and I-phenotype tumors than in HGM-positive tumors, MUC6-positive tumors, CD10-negative tumors and G-phenotype tumors (32.7% *vs* 7.1%, $P < 0.0001$; 27.8% *vs* 14.0%, $P = 0.0182$; 37.3% *vs* 10.4%, $P < 0.0001$; and 38.5% *vs* 9.5%, $P = 0.0017$, respectively). The frequency of MSI was significantly higher in MUC6-positive tumors, CD10-negative tumors and G-phenotype tumors than in MUC6-negative tumors, CD10-positive tumors and I-phenotype tumors (24.8% *vs* 6.7%, $P = 0.0009$; 22.2% *vs* 8.0%, $P = 0.0143$; and 28.6% *vs* 9.6%, $P = 0.0353$, respectively).

CONCLUSION: The tumor differentiation phenotype is closely related to tumor invasion and genetic alterations in gastric differentiated-type tumors.

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Key words: Gastric carcinoma; Tumor differentiation phenotype; *APC*; *p53*; Microsatellite instability

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INTRODUCTION

Gastric carcinoma is histologically classified into two types, differentiated- and undifferentiated-type or intestinal- and diffuse-type, based on the prevalent gland formation[1,2]. The pathogenesis and genetic alterations leading to these two distinct types of adenocarcinoma are generally considered different. Previous molecular genetic studies have shown that carcinogenesis is a multistep process characterized by accumulating genetic alterations[3,4]. *K-ras* mutations, *c-erbB-2* amplification, *APC* mutations, allelic loss of the *Bcl*-II and *DCC* loci, and microsatellite instability (MSI) are preferentially associated with differentiated-type carcinoma[5,6]. Conversely, the reduced or absent expression of *E-cadherin* and *K-sam* amplification are unique to undifferentiated-type carcinoma. The abnormal expres-

sion and amplification of *Met*, the inactivation of the *p53* tumor suppressor gene, the abnormal transcription of *CD44*, and the loss of telomerase are common events in both types[3,4,7]. Recently, several authors have reported that gastric carcinomas can be classified as having either a gastric- (G-), gastric and intestinal mixed- (GI-) or intestinal- (I-) phenotype, depending on the immunopositivity of human gastric mucin (HGM), MUC6, MUC2 and CD10 stainings[8-10]. HGM, MUC6, MUC2 and CD10 are specifically expressed in gastric surface mucous cells, pyloric gland cells, intestinal goblet cells of the mature gastrointestinal tract, and the brush border of intestinal epithelial cells, respectively. We previously reported that G-phenotype tumors account for 27.7% of differentiated tumors, and are often referred to as intestinal-type tumors according to Lauren, while I-phenotype tumors account for 10.1% of undifferentiated tumors[8]. The phenotypic marker expression of tumors is conventionally thought to imitate that of the tissue of origin[11-13]. Thus, the above data suggest that gastric carcinomas can occur in various types of gastric mucosa, although differentiated tumors are generally thought to arise from gastric mucosa with intestinal metaplasia while undifferentiated tumors are thought to arise from ordinary gastric mucosa without intestinal metaplasia[1,2]. Recently, G-phenotype tumors have been associated with a poorer outcome and greater malignant potential, compared with tumors of other phenotype[8-10,14-17]. Therefore, this phenotypic classification is useful not only for investigating the tumorigenesis of gastric carcinoma, but also for evaluating tumor aggressiveness. Phenotypic expression in gastric neoplasm has also been suggested to depend on genetic changes[18]. However, there are conflicting opinions regarding the relations between tumor differentiation phenotype and tumor invasion or genetic alterations in gastric differentiated-type tumors.

In the present study, we examined the tumor differentiation phenotype, mutations in *APC* and *p53* genes, and the MSI status in 48 gastric adenomas and 171 differentiated-type adenocarcinomas to clarify the relations between tumor differentiation phenotype and tumor invasion or genetic alterations in gastric differentiated-type tumors.

MATERIALS AND METHODS

Patients

The present series consisted of 48 patients with gastric adenoma having undergone an endoscopic biopsy or an endoscopic mucosal resection and 171 patients with differentiated-type gastric adenocarcinoma having undergone surgical resection between 2001 and 2003 at Showa University Hospital. Written informed consent was obtained from all the patients before they were included in the present study.

Histological review

The specimens were fixed with 10% buffered formalin and embedded in paraffin. Consecutive sections (4 μm thick) were stained with hematoxylin and eosin (HE) and immunohistochemical stains for the histopathological examination. The intramucosal neoplastic lesions were histologically classified according to the general rules established by the Vienna classification[19]. Forty-eight adenomas (35 low-grade adenomas and 13 high-grade adenomas), 132 differentiated-type, early-stage carcinomas (80 intramucosal carcinomas and 52 carcinomas with invasion to the submucosal layer), and 39 differentiated-type, advanced-stage carcinomas (13 carcinomas with invasion to the muscularis propria layer, 15 carcinomas with invasion to the subserosa, and 11 carcinomas with invasion to the serosa) were identified.

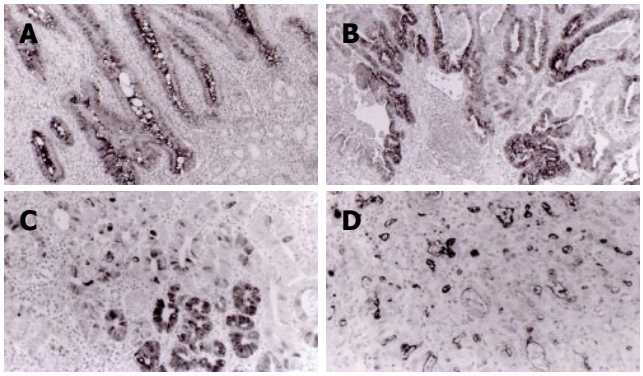


Figure 1 Immunohistochemical analysis of tumor differentiation phenotype in gastric tumor. **A**: Human gastric mucin (HGM) (45M1, × 200); **B**: MUC6 (CLH5, × 100); **C**: MUC2 (Ccp58, × 200); **D**: CD10 (56C6, × 200).

Analysis of tumor differentiation phenotype

The following mouse monoclonal antibodies were used: 45M1 (Novocastra Laboratories Ltd, UK) diluted 1:50 to detect HGM, CLH5 (Novocastra Laboratories Ltd, UK) diluted 1:50 to detect MUC6 glycoprotein, Ccp58 (Novocastra Laboratories Ltd, UK) diluted 1:100 to detect MUC2 glycoprotein, and 56C6 (Novocastra Laboratories Ltd, UK) diluted 1:40 to detect CD10 glycoprotein expression. 45M1 and CLH5 were examined as gastric-phenotype markers, and Ccp58 and 56C6 were examined as intestinal-phenotype markers. 45M1 could recognize the mucin epitope located in the peptide core of HGM, synonymous with MUC5AC. This antibody is known to react with surface foveolar cells in the stomach[20] (Figure 1A). MUC6 glycoprotein is expressed in mucous cells in the neck zone of oxyntic mucosa and in antral glands[21,22] (Figure 1B). MUC2 glycoprotein, also known as the "intestinal-mucin related protein antigen", is an intestinal apomucin that is known to be expressed in the supranuclear area of goblet cells in regions showing intestinal metaplasia in the stomach[22,23] (Figure 1C). CD10 glycoprotein is a 100-kDa cell metalloendopeptidase that inactivates a variety of biologically active peptides and is known to be expressed on the brush border of intestinal epithelial cells as well as in the germinal centers of lymphoid follicles[24] (Figure 1D). The avidin-biotin-peroxidase complex immunohistochemical method was used for all immunohistchemical studies according to a previously described protocol[25].

With regard to the evaluations of HGM, MUC6, MUC2, and CD10 staining, distinct staining in more than 5% of the tumor cells was recorded as positive immunore-

	Gastric-phenotype markers HGM - MUC6 +	
Intestinal-phenotype markers MUC2 -		Gastric-phenotype
Intestinal-phenotype markers CD10 +	Intestinal-phenotype	Gastric and intestinal mixed-phenotype

Figure 2 Classification of tumor differentiation phenotype. The phenotypes were classified into three groups (gastric-phenotype, intestinal-phenotype, gastric and intestinal mixed-phenotype) according to the combination of the expression of human gastric mucin (HGM), MUC6, MUC2 and CD10.

activity for the relevant marker. These immunohistochemical methods were used to classify the tumors into three different phenotypes: tumors with gastric phenotypic cells accounting for more than 5% of their cell population were classified as gastric- (G-) phenotype tumors, those with intestinal phenotypic cells accounting for more than 5% of their cell population were classified as intestinal- (I-) phenotype tumors, and those with both gastric and intestinal phenotypic cells accounting for more than 5% of their cell population were classified as gastric and intestinal mixed- (GI-) phenotype tumors as previously described[8,9,26] (Figure 2).

DNA extraction

Serial sections (10 μm thick) of formalin-fixed, paraffin-embedded tumor and normal mucosa were stained with HE and precisely microdissected under microscopic visualization using a PixCell laser capture microdissection system (Arcus Engineering, Mountain View, CA) to avoid DNA contamination from inflammatory or stromal cell nuclei and the DNA of cancerous cells inconsistent with the overt differentiation phenotype of the tumor. Genomic DNA was extracted from the microdissected tissue as previously described[27].

Mutation analysis of APC and p53

APC mutations in the exon 15, codons 1260-1596 region were detected by fluorescence-based PCR-single-strand conformation polymorphism analysis (PCR-SSCP) using an automated DNA sequencer as previously described[28]. This region was amplified by 4 parts using the following primer sets: 5'-CAGACTTATTGTGTAGAAGA-3' and 5'-CTCCTGAAGAAAATTCAACA-3', 5'-AGGGTTC-TAGTTTATCTTCA-3' and 5'-TCTGCTTGGTGGCAT-GGTTT-3', 5'-AACCATGCAGTGGAATGGTA-3' and 5' -AAATGGCTCATCGAGGCTCA-3', 5'-ACTCCAGAT-GGATTTTCTTG-3' and 5'-GGCTGGCTTTTTTGCTT-TAC-3'. The 5'-terminals of the primers were labeled with Cy5 dye. DNA fragments were amplified by PCR from 2 μL of 50 μL of extracted DNA. PCR was performed at 94℃ for 1 min followed by 40 cycles at 94℃ for 1 min, at 58℃ for 1 min, and at 72℃ for 2 min. Mutations were detected by electrophoresis using the ALFexpress DNA sequencer (Amersham Biosciences, NJ, USA) with TME buffer (30 mmol/L Tris-HCl, 36 mmol/L MES, 1 mmol/L EDTA; pH 6.8) at 20℃, 25℃, and 30℃. The detected peaks were analyzed using the Fragment Manager program (Amersham Biosciences).

p53 mutations in the exon 5-8 region were detected by fluorescence-based PCR-SSCP using capillary electrophoresis[28]. The nucleotide sequences of the forward and reverse primers were as follows: 5'-TTCACTTGT-GCCCTGACTTTC-3' and 5'- CTCTCCAGCCCCAGCT-GCTC-3' for exon 5, 5'-ATTCCTCACTGATT-GCTCTT-3' and 5'-TCCTCCCAGAGACCCCAGTT-3' for exon 6, 5'-ACAGGTCTCCCCAAGGCGCA-3' and 5'-TGCAGGGTGGCAAGTGGCT-3' for exon 7, 5' -GGTAGGACCTGATTTCCTTACTGCC-3' and 5' -CCCTTGGTCTCCTCCACCGCTTCTTG-3' for exon 8. The 5'-terminals of the primers for p53 were labeled with FAM. DNA fragments were amplified by PCR from 2 μL of 50 μL of extracted DNA. Mutations were detected by electrophoresis using a 3100 genetic analyzer (Applied Biosystems, CA, USA) with 2 × TME buffer (60 mmol/L Tris-HCl, 72 mmol/L MES, 2 mmol/L EDTA). The precise electrophoresis conditions have been described elsewhere[29]. The peak pattern was analyzed using QUISCA software, established by Higasa *et al*[30] and kindly provided by Dr. K Hayashi. The nucleotide sequences of DNA fragments with shifted peaks were determined using a genetic analyzer 310 with a BigDye Terminator (Applied Biosystems).

Microsatellite instability analysis

Microsatellite instability was detected using five microsatellite markers: BAT-25, BAT-26, D2S123, D5S346, and D17S250. The primers and PCR conditions have been described elsewhere[31]. The 5'-terminals of the forward primers were labeled with FAM. PCR was performed at 94℃ for 1 min followed by 35 cycles at 95℃ for 30 s, at 50℃ for 1 min, and at 70℃ for 1 min. The samples were capillary electrophoresed on an ABI 3100 genetic analyzer using Genescan analysis software (Applied Biosystems). An allelic shift (MSI) in a microsatellite marker was identified by the presence of at least one additional band in the tumor's DNA that was not present in the control DNA. A specimen was defined as MSI-high (MSI-H) when at least two markers showed instability, MSI-low (MSI-L) when only one marker showed instability, and microsatellite stable (MSS) when none of the markers exhibited MSI[32].

Statistical analysis

The data were analyzed using Student *t*-test or Mann-Whitney *U*-test and chi square test or Fisher' exact test, as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

HGM, MUC6, MUC2 and CD10 expressions, tumor differentiation phenotype, and genetic alterations in 219 gastric tumors

The expression of HGM, MUC6, MUC2, and CD10 was demonstrated in 112 (51.1%), 129 (58.9%), 163 (74.4%) and 75 (34.2%) of the 219 gastric tumors, respectively. Taking into account the observed combinations of these phenotypic markers, the 219 tumors were classified into 42 G-phenotype tumors (19.2%), 125 GI-phenotype

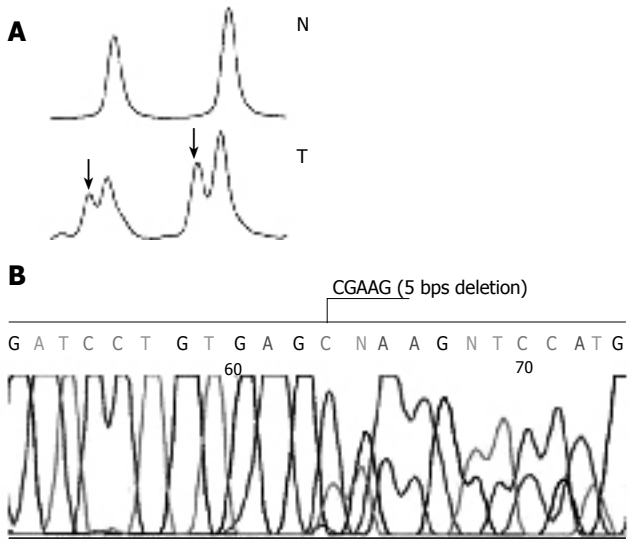


Figure 3 Mutation analysis of the APC gene in gastric tumour. **A**: PCR-single-strand conformation polymorphism (PCR-SSCP) analysis shows shift peak (arrowhead) as compared with the control normal DNA; **B**: DNA sequence analysis reveals frameshift mutation (CGAAG, 5 bps deletion) at codons 1321 of the APC gene. T: Tumor DNA, N: Normal DNA.

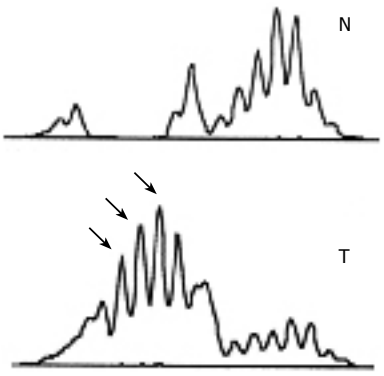


Figure 4 Microsatellite instability (MSI) using marker BAT-26 shows a different allelic shift peak (arrowhead) as compared with the control normal DNA. T: Tumor DNA, N: Normal DNA.

Table 1 Phenotypic expressions, tumor differentiation phenotype and genetic alterations in gastric adenoma and carcinoma *n* (%)

Phenotypic expressions, tumor differentiation phenotype &genetic alterations	Adenoma (*n* = 48)	Early carcinoma (*n* = 132)	Advanced carcinoma (*n* = 39)	Early and advanced carcinoma (*n* = 171)
HGM				
Negative	43 (89.6)	49 (37.1)[d]	15 (38.5)[d]	64 (37.4)[d]
Positive	5 (10.4)	83 (62.9)[d]	24 (61.5)[d]	107 (62.6)[d]
MUC6				
Negative	29 (60.4)	48 (36.4)[b]	13 (33.3)[a]	61 (35.7)[b]
Positive	19 (39.6)	84 (63.6)[b]	26 (66.7)[a]	110 (64.3)[b]
MUC2				
Negative	6 (12.5)	35 (26.5)	15 (38.5)[a]	50 (29.2)[a]
Positive	42 (87.5)	97 (73.5)	24 (61.5)[a]	121 (70.8)[a]
CD10				
Negative	16 (33.3)	100 (75.8)[d]	28 (71.8)[c]	128 (74.9)[d]
Positive	32 (66.7)	32 (24.2)[d]	11 (28.2)[c]	43 (25.1)[d]
Tumor differentiation phenotype				
G-phenotype	2 (4.2)	30 (22.7)[d]	10 (25.6)[d]	40 (23.4)[d]
GI-phenotype	19 (39.6)	82 (62.1)[d]	24 (61.5)[d]	106 (62.0)[d]
I-phenotype	27 (56.3)	20 (15.2)[d]	5 (12.8)[d]	25 (14.6)[d]
APC				
Negative	29 (60.4)	110 (83.3)[b]	37 (94.9)[c]	147 (86.0)[d]
Positive	19 (39.6)	22 (16.7)[b]	2 (5.1)[c]	24 (14.0)[d]
p53				
Negative	46 (95.8)	99 (75.0)[b]	27 (69.2)[b]	126 (73.7)[b]
Positive	2 (4.2)	33 (25.0)[b]	12 (30.8)[b]	45 (26.3)[b]
MSI status				
MSS	40 (83.3)	107 (81.1)	34 (87.2)	141 (82.5)
MSI-L	4 (8.3)	16 (12.1)	2 (5.1)	18 (10.5)
MSI-H	4 (8.3)	9 (6.8)	3 (7.7)	12 (7.0)

HGM: Human gastric mucin, [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.0001$ *vs* adenoma.

tumors (57.1%) and 52 I-phenotype tumors (23.7%). *APC* mutations were detected in 43 (19.6%) of the 219 tumors. Of these mutations, 30 (69.8%) were frameshift mutations (20 deletions and 10 insertions) (Figure 3) and 13 (30%) were point mutations (3 missense and 10 nonsense). *p53* mutations were detected in 47 (21.5%) of the 219 tumors. Of these mutations, 38 (80.9%) were point mutations (35 missense and 3 nonsense) and 9 (19.1%) were frameshift mutations (9 deletions). MSI was observed in 38 (17.4%) of the 219 tumors (Figure 4). MSI-L and MSI-H tumors were observed in 22 (10.0%) and 16 (7.3%) of the 219 tumors, respectively.

Tumor differentiation phenotype and genetic alterations in gastric adenoma and carcinoma

The tumor differentiation phenotype, presence of *APC* or *p53* mutations, and the MSI-status in gastric adenomas, early and advanced carcinomas examined in this study are shown in Table 1. Gastric adenomas were significantly associated with CD10 expression, I-phenotype and *APC* mutations compared with carcinomas (66.7% *vs* 25.1%, $P < 0.0001$; 56.3% *vs* 14.6%, $P < 0.0001$; and 39.6% *vs* 14.0%, $P < 0.0001$, respectively), and inversely associated with HGM and MUC6 expression and *p53* mutations (10.4% *vs* 62.6%, $P < 0.0001$; 39.6% *vs* 64.3%, $P = 0.0030$; 4.2% *vs* 26.3%, $P = 0.0010$, respectively). There were no differences in any of the variables listed in Table 1 between early and advanced carcinomas.

Relations between HGM, MUC6, MUC2, or CD10 expressions and genetic alterations

The relations between the expressions of HGM, MUC6, MUC2, or CD10 and *APC* mutations, *p53* mutations, and the MSI status in gastric tumors are shown in Table 2. The frequency of *APC* mutation was significantly higher in HGM-negative, MUC6-negative and CD10-positive tumors than in HGM-positive, MUC6-positive and CD10-negative tumors (32.7% *vs* 7.1 %, $P < 0.0001$; 27.8% *vs* 14.0%, $P = 0.0182$; and 37.3% *vs* 10.4%, $P = 0.0398$, respectively). The frequency of MSI was significantly higher in MUC6-positive and CD10-negative tumors than in MUC6-negative and CD10-positive tumors (24.8% *vs* 6.7%, $P = 0.0009$ and 22.2% *vs* 8.0%, $P = 0.0143$, respectively). There were no significant differences in the frequency of *p53* mutations according to the expressions of HGM, MUC6, MUC2 or CD10.

Table 2 Relationship between expressions of HGM, MUC6, MUC2 and CD10, and genetic alterations in 219 gastric tumors *n* (%)

	HGM		MUC6		MUC2		CD10	
	Negative (*n* = 107)	Positive (*n* = 112)	Negative (*n* = 90)	Positive (*n* = 129)	Negative (*n* = 56)	Positive (*n* = 163)	Negative (*n* = 144)	Positive (*n* = 75)
APC	35 (32.7)	8 (7.1)[c]	25 (27.8)	18 (14.0)[a]	10 (17.9)	33 (20.2)	15 (10.4)	28 (37.3)[c]
p53	21 (19.6)	26 (23.2)	19 (21.1)	28 (21.7)	10 (17.9)	37 (22.7)	28 (19.4)	19 (25.3)
MSI status								
MSI-L	12 (11.2)	12 (10.7)	4 (4.4)	20 (15.5)	7 (12.5)	17 (10.4)	17 (11.8)	6 (8.0)
MSI-H	7 (6.5)	7 (6.3)	2 (2.2)	12 (9.3)	5 (8.9)	9 (5.5)	15 (10.4)	0 (0)
Total	19 (17.8)	19 (17.0)	6 (6.7)	32 (24.8)[b]	12 (21.4)	26 (16.0)	32 (22.2)	6 (8.0)[a]

HGM: Human gastric mucin, [a]$P < 0.05$, [b]$P < 0.001$, [c]$P < 0.0001$ *vs* negative.

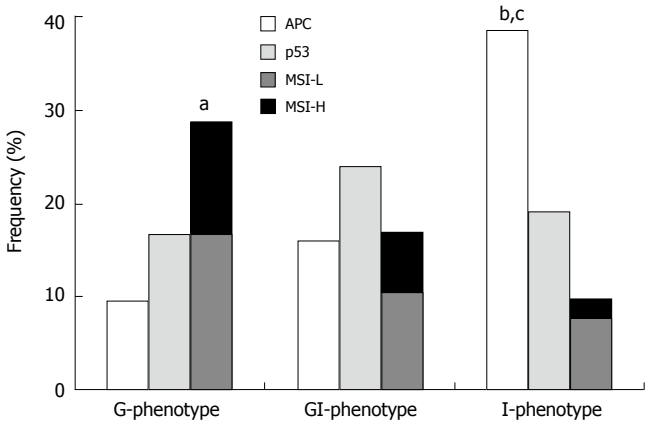


Figure 5 Frequency of *APC*, *p53* mutation and MSI in gastric- (G-), gastric and intestinal mixed- (GI-), and intestinal- (I-) phenotype tumors. [a]$P = 0.0353$ (G-phenotype *vs* I-phenotype, 28.6% *vs* 9.6%), [b]$P = 0.0017$ (I-phenotype *vs* G-phenotype, 38.5% *vs* 9.5%), [c]$P = 0.0022$ (I-phenotype *vs* GI-phenotype, 38.5% *vs* 16.0%).

Relations between tumor differentiation phenotype and genetic alterations

The relations between tumor differentiation phenotype and *APC* mutations, *p53* mutations and the MSI-status in gastric tumors are shown in Figure 5. The frequency of *APC* mutations was significantly higher in I-phenotype tumors than in G- phenotype and GI-phenotype tumors (38.5% *vs* 9.5% and 16.0%, $P = 0.0017$ and $P = 0.0022$, respectively). The frequency of MSI was significantly higher in G-phenotype tumors than in I-phenotype tumors (28.6% *vs* 9.6%, $P = 0.0353$). There were no significant differences in the frequency of *p53* mutations among the G-, GI- and I-phenotypes.

DISCUSSION

Our present results revealed that gastric adenomas were significantly associated with CD10 expression, I-phenotype and *APC* mutation and inversely associated with HGM and MUC6 expression and *p53* mutations, compared with early and advanced carcinomas. Gastric adenomas were also significantly associated with MUC2 expression, compared with advanced carcinomas. Therefore, gastric adenomas are clearly different from early and advanced carcinomas in terms of the tumor differentiation phenotype and genetic alterations.

With respect to the difference in the tumor differentiation phenotype between gastric adenomas and carcinomas, our present results revealed that more than half of gastric adenomas exhibited I-phenotype and the incidence of I-phenotype tumor was significantly higher in gastric adenomas than in early and advanced carcinomas. On the other hand, the majority of early and advanced carcinomas exhibited a G- or GI-phenotype. These findings indicate that the tumor differentiation phenotype is related to tumor invasion in gastric differentiated-type tumors. Recently, several reports have demonstrated distinct differences in the biological behavior of tumors with different phenotypic markers[8-10,14-18,26]. Endoh *et al*[34] reported that low positivity for Ki-67 staining along the mature collagenous stromal reaction in the invasive area can be seen in differentiated-type carcinomas of I-phenotype, reflecting the slow growth of this tumor. We have previously reported that patients with G-phenotype tumors have a significantly poorer prognosis than those with I-phenotype tumors in patients with advanced gastric carcinoma[8].

In the present study, gastric adenomas were significantly associated with *APC* mutations and inversely associated with *p53* mutations, compared with those in early and advanced carcinomas. MSI is a common event in both gastric adenomas and differentiated-type carcinomas. The absence of sequential accumulations of genetic alterations between gastric adenomas and early or advanced carcinomas suggests that gastric differentiated-type tumors with *APC* mutations, especially gastric adenomas, may have a low invasive potential. Lee *et al*[31] suggested that gastric adenomas without *APC* mutations have a different biological behavior from those with *APC* mutations and are precursors of differentiated-type carcinoma. Distinct differences in the tumor differentiation phenotype and genetic alterations between gastric adenomas and differentiated-type carcinomas suggest that the adenoma-carcinoma sequence, typically seen in colorectal carcinoma, is not be a major pathway in tumorigenesis of gastric differentiated-type tumor. Furthermore, our present findings also suggest that the tumor differentiation phenotype and genetic alterations are related to tumor aggressiveness in gastric differentiated-type tumors.

The relations between tumor differentiation phenotype and genetic alterations have not been examined in any large study on gastric adenoma and early differentiated-type carcinoma. Our present results revealed that MSI was

significantly associated with MUC6 expression and G-phenotype, and inversely associated with CD10 expression. On the other hand, *APC* mutation was significantly associated with CD10 expression and I-phenotype, and inversely associated with expressions of HGM and MUC6, suggesting that the tumor differentiation phenotype is closely related to genetic alterations in gastric differentiated-type tumors. Differentiation of gastric epithelial cells, especially pyloric gland cells, may be related to MSI, while differentiation of intestinal epithelial cells, especially absorptive cells, may be related to *APC* mutations. To the best of our knowledge, this is the first report describing associations between genetic alterations, such as *APC* mutations and MSI, and the expressions of HGM, MUC6, MUC2 or CD10 in gastric differentiated-type tumors.

The differences in the biological behavior of tumors with different phenotype as described above suggest that the action of different genetic alterations depends on the tumor differentiation phenotype of gastric tumors. Kabashima *et al*[10] reported that G-phenotype tumors can potentially degrade the extracelluar matrix through the overexpression of matrix metalloproteinases, compared with I-phenotype tumors. Shibata *et al*[17] reported that the apototic index/proliferative index ratio is significantly lower in G-phenotype tumors than in I-phenotype tumors. Endoh *et al*[18] have detected *E-cadherin* mutations in 21% of differentiated-type carcinomas with a G-phenotype, although this mutation is generally considered to be involved in undifferentiated-type but not differentiated-type carcinomas. Our present findings show that the tumor differentiation phenotype is closely related to tumor invasion and genetic alterations in gastric differentiated-type tumors. Previous molecular genetics studies have shown that gastric tumorigenesis is a multistep process with accumulation of genetic alterations[3,4]. Therefore, the previous data and our present findings suggest that different genetic pathways according to the tumor differentiation phenotype might play a role in the tumorigenesis of the gastric differentiated-type tumor, leading to their different biological behavior.

In conclusion, the tumor differentiation phenotype is closely related to tumor invasion and genetic alterations in gastric differentiated-type tumors.

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GASTRIC CANCER

Effects of recombinant human growth hormone on growth of human gastric carcinoma xenograft model in nude mice

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Abstract

AIM: To study effects of recombinant human growth hormone (rhGH) on growth of a human gastric carcinoma cell *in vivo*.

METHODS: Experimental mice were divided into control group, rhGH group, oxaliplatin (L-OHP) group and rhGH+L-OHP group. Cultured human gastric carcinoma cells BGC823 were inoculated into right axilla of nude mice and carcinoma xenograft model was established successfully. Inhibitory rate of xenograft tumor growth was estimated by measuring tumor volume; expression of proliferating cell nuclear antigen (PCNA), Bax and Bcl-2 proteins of xenograft tumor was detected using immunohistochemical S-P method.

RESULTS: Tumor growth inhibitory rate, the positive expression rate of PCNA, Bax and Bcl-2 were 49.3%, 58.2%, 65.2% and 59.2% in rhGH+L-OHP group respectively; 46.6%, 62.5%, 59.7% and 64.7% in L-OHP group; 5.0%, 82.7%, 23.2% and 82.2% in rhGH group and 0, 77.8%, 23.5% and 80.3% in control group. There was significant difference between rhGH+L-OHP group (or L-OHP group) and control group or rhGH group ($P < 0.05$), whereas there were no significant differences ($P > 0.05$) between L-OHP group and rhGH+L-OHP group and between rhGH group and control group.

CONCLUSION: rhGH does not accelerate the proliferation of human gastric cancer cell *in vivo*.

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Key words: Human growth hormone; Stomach neoplasm; Nude mice

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INTRODUCTION

Recombinant human growth hormone (rhGH) can reverse many nutritional and metabolic abnormalities associated with severe catabolic states. In clinical studies rhGH has been shown to promote protein synthesis, improve nitrogen balance, accelerate wound healing[1-6], maintain host immune function and alleviate postoperation fatigue syndrome (POF)[7]. GH has become available for clinical use. It was reported that rhGH enhanced positive nitrogen balance in metabolic recuperation of postoperative patients who suffered from malignant tumor[8,9]. However, it is still controversial[10-12] whether rhGH should be applied in postoperative tumor patients since hGH promoted the proliferation of normal cells, as well as tumor cells. In the present study we investigated the effects of rhGH on human gastric cancer cell line BGC823 *in vivo* by inducing carcinoma xenograft model in nude mice, in an effort to clarify whether rhGH can be applied in postoperative patients with gastric cancer for metabolic intervention.

MATERIALS AND METHODS

Materials

B*ALB/C* nude mice were obtained from Shanghai Pharmaceutical Institute of Chinese Academy of Sciences. The mice were 6 wk old, with weights ranging between 12-22 g. Human gastric cancer cell line BGC823 was supplied by the Cell Bank of Shanghai Cell Biology Institute of Chinese Academy of Sciences. rhGH (Saizen) was supplied by Serono (Switzerland). Oxaliplatin (L-OHP) was purchased from Henrui Pharmaceutical Company, Jiangsu, China. Immuno-histochemical reagents, including proliferating cell nuclear antigen (PCNA), Bax and Bcl-2 were obtained from Maixin Reagents Company, Fujian, China.

Methods

At logarithmic growth phase, the cells of human gastric

Table 1 Volume and inhibitory rate of xenograft tumor growth (n = 6, mean ± SD)

Group	Volume (cm^3)	Inhibitory rate (%)
Control	0.406 ± 0.138	0
rhGH	0.383 ± 0.136	5
L-OHP	0.215 ± 0.087[a]	46.6
rhGH+L-OHP	0.208 ± 0.102[a]	49.3

[a]P < 0.05 *vs* control or rhGH.

cancer cell line BGC823 were digested by trypsin. Then the activity of the cells was examined (*Via* = 98.6%) and the cells were counted in hemocytometer using Trypan blue exclusion. The density of single cell suspension was adjusted to 1×10^{10}/L for further use.

Cell suspension (0.2 mL) was inoculated subcutaneously into right axilla of nude mice and all of carcinoma xenograft models were established within 3-7 d. Then the volume of tumor was measured. The experimental mice were randomly divided into 4 groups: control group, rhGH group, L-OHP group and rhGH+L-OHP group, 6 in each according to the drugs they received. From the 8th day of inoculation, the following drugs were administrated for 6 consecutive days: normal saline(NS) was subcutaneously injected in control group, 0.1 mL/d; rhGH group with rhGH, 2 IU/kg per day; L-OUP group with L-OHP by celiac injection, 1.3 mg/kg per day; and rhGH+L-OHP group with both rhGH and L-OHP of the same dosage as rhGH group and L-OHP group, respectively.

Immunohistochemistry

The mice were killed on the 7 d of drug administration and inhibitory rate of xenograft tumor growth was estimated by measuring tumor volume and expressions of PCNA, Bax and Bcl-2 proteins were detected by using immunohistochemical technique. Definition of positive PCNA cells was that cytoblast was nigger-brown stained and that for positive Bax and Bcl-2 cells was brown yellow stained in cytoplasm and cell membrane.

Statistical analysis

Data were expressed as mean ± SD and analyzed by variance analysis with SPSS11.0. Statistical significance was considered at $\alpha = 0.05$.

RESULTS

Inhibitory rate of xenograft tumor growth

Length(a) and breadth(b) of xenograft tumor were measured and the volume was counted using the formula: $V = ab^2/2$, as Oscieka described[13]. Xenograft tumor grew more slowly and tumor growth inhibitory rate was larger in rhGH+ L-OHP group and L-OHP group compared with control group and rhGH group ($P < 0.05$), whereas there was no significant difference between control group and rhGH group or between L-OHP and rhGH+L-OHP groups (Table 1). Tumor growth inhibitory rate (%) = [(volume in control group-volume in experiment group)/ volume in control group] × 100%

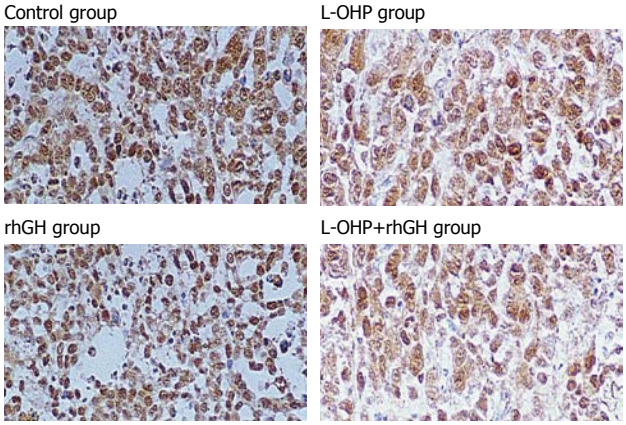


Figure 1 Positive expression of PCNA of human gastric cancer cell (SABC × 100).

Detection of PCNA, bax and Bcl-2 proteins using immunohistochemical technique

Positive expression of PCNA of human gastric cancer: There were more cells whose cytoblasts were nigger-brown stained in control group and rhGH group compared with L-OHP group and rhGH+L-OHP group.

Positive expression of Bcl-2 of human gastric cancer: There were more cells whose cytoplasm and cell membrane were brown yellow stained in control group and rhGH group compared with L-OHP group and rhGH+L-OHP group. In contrast, positive expression of Bax was seen more in L-OHP group and rhGH+L-OHP group (Figures 1, 2, and 3).

PCNA and Bcl-2 expressions were obviously decreased, while Bax distinctly increased in rhGH+L-OHP group and L-OHP group compared with control group and rhGH group ($P < 0.05$), but there was no significant difference between control group and rhGH group or between L-OHP and rhGH+L-OHP group (Table 2).

DISCUSSION

Nude mouse was a mutant mouse found and cultured in 1966. Because it has no thymus and completely lacks lymphocyte function and has no repellent reaction to heterogeneous transplant, it is especially adapted to animal heterogeneous transplant and human tumor heterogeneous transplant. In addition, karyotype, histological form and tumor biological characters of transplant tumor is the same as original tumor. Therefore, tumor model of nude mice is an ideal model for studying human malignant tumor. In our study, tumor xenograft model was successfully established through inoculation of human gastric carcinoma cells BGC823 into right axilla of nude mice. All tumor xenograft grew obviously.

In recent years, many studies reported that rhGH did not promote tumor growth[14-17]. Researchers from home and abroad found that rhGH accelerated improvement of patients by tentatively applying rhGH to patients with gastrointestinal malignant tumor after operation for metabolic intervention. rhGH did not promote tumor

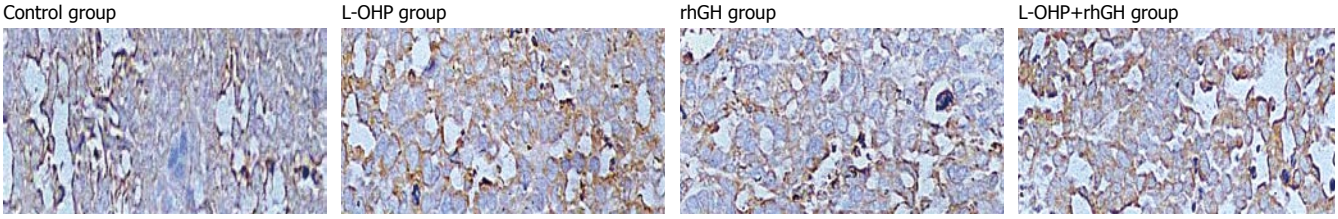


Figure 2 Positive expression of Bax of human gastric cancer cell (SABC × 200).

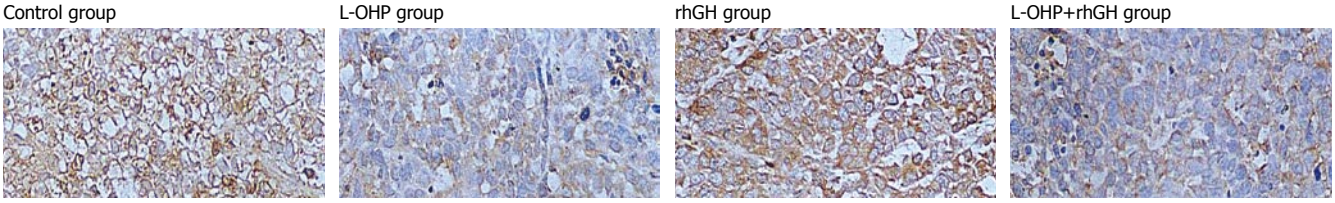


Figure 3 Positive expression of Bcl-2 of human gastric cancer cell (SABC × 100).

Table 2 Percentages of positive cells of PCNA, Bax and Bcl-2 expression

Group	PCNA	Bax	Bcl-2
Control	77.8 ± 6.3	23.5 ± 4.3	80.3 ± 8.9
rhGH	82.7 ± 9.4	23.2 ± 5.7	82.2 ± 7.4
L-OHP	62.5 ± 7.2[a]	59.7 ± 12.8[a]	64.7 ± 6.2[a]
rhGH+L-OHP	58.2 ± 5.2[a]	65.2 ± 11.9[a]	59.2 ± 9.2[a]

[a]$P < 0.05$ *vs* control or rhGH.

growth and metastasis in all their reported cases. Juergen *et al*[18] showed that postoperative treatment with rhGH in short term did not promote recurrence of tumor in 75 patients with gastrointestinal malignant tumor. Others carried out short-term nutritional treatment with rhGH in patients with tumor completely excised or other anticancer therapy[19].

In the present study, in comparison of rhGH+L-OHP and L-OHP groups with control and rhGH groups, PCNA and Bcl-2 apparently decreased and Bax obviously increased ($P<0.05$), while there was no significant difference between control group and rhGH group or between L-OHP and rhGH+L-OHP groups. It suggests that rhGH did not increase gastric cancer cell growth. In addition, tumor grew more slowly and tumor inhibitory rate was higher in L-OHP and rhGH+L-OHP groups, compared with control group and rhGH group, whereas there were no distinct differences between control group and rhGH group or between L-OHP and rhGH+L-OHP groups. These results also indicate that rhGH did not accelerate gastric cancer growth.

This study showed rhGH did not accelerate growth of a human gastric carcinoma xenograft model in nude mice. The results coincide with Fiebig's study that rhGH did not promote tumor (renal cancer and lung cancer) growth in nude mice[20] and also our former experiment *in vitro*[21]. In addition, our former study results showed tumor inhibitory rate did not change correspondingly with increase of the dose of rhGH. On the other hand, Blanck *et al*[22] proved rhGH did not promote liver cancer and renal cancer growth in rat tumor model. The mechanism that rhGH does not promote tumor growth is unknown. The possible mechanisms are (1) rhGH enhanced immunity of body[23]. Some researchers found GH and insulin-like growth factor-1 (IGF-1) increased the activity of natural killer cell (NK)[24,25]; (2) hGH directly or indirectly stimulate cell toxic effect[23]; (3) GH receptor expression reduced in tumor cell[22,26]; (4) IGF-1 and insulin-like growth factor binding protein-3 (IGFBP-3) were increased because of therapy of GH and IGFBP counteracted proliferation effect of IGF-I. Furthermore, IGFBP accelerated apoptosis independently of IGF-1[27]. Moreover, IGFBP-3 has been reported to inhibit the development of colonic tumors in experimental models and may hold promise as an adjuvant therapy for patients with neoplasm[28].

To sum up, our study demonstrates that postoperative treatment with rhGH in short term does not promote gastric cancer growth in patients with gastric cancer and it is safe using rhGH with chemotherapeutics.

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GASTRIC CANCER

Single-tube-genotyping of gastric cancer related SNPs by directly using whole blood and paper-dried blood as starting materials

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Abstract

AIM: To demonstrate an inexpensive method for typing gastric cancer related single nucleotide polymorphisms (SNPs) using whole blood or paper-dried blood as starting materials.

METHODS: PCR amplification is directly carried out from the whole blood or paper-dried blood sample without any DNA extraction step. Before PCR, a blood sample, four primers, and all of biological reagents necessary for PCR were added at a time; After PCR, the amplified products were directly separated by slab gel electrophoresis or microchip CE without any purification. SNP typing was performed by tetra-primer PCR with two inner primers specific to each allele and two outer primers defining the length of allele-specific amplicons. Genotypes were directly discriminated by the size of amplicons specific to each allele, thereby avoiding any post-PCR process.

RESULTS: Using a special PCR buffer, inhibitory substances in blood (including the anticoagulant in blood) and filter paper were effectively suppressed; a "true" single-tube-genotyping is thus realized. We successfully determined genotypes IL-1B-511 and IL-1B-31 polymorphisms at the gene IL-1B by using whole-blood and paper-dried blood samples as starting materials respectively. The method is so sensitive that 0.5-1.0 μL of blood sample is enough to give a satisfactory typing results. The genotyping results were confirmed by RFLP-PCR using purified genome DNA, indicating that amplification specificity was not affected by inhibitory components (including coagulants) in blood or filter paper.

CONCLUSION: Compared with SNP typing methods based on purified DNA, the proposed method is labor-saving, simple, inexpensive, and less cross-contaminated. It is promising to use this method to type other SNPs.

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Key words: Gastric cancer; Whole blood; Paper-dried blood; Tetra-PCR; SNP; IL-1B-31; IL-1B-511

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INTRODUCTION

Gastric cancer is a complex disease with a high mortality rate[1-3], but the recent success of the Human Genome Project[4] as well as the identification of vast numbers of single nucleotide polymorphisms (SNPs)[5] enables us to prevent or alleviate the disease by detecting the potential disease-susceptibility alleles and diallelic markers. It was reported that cytokine IL-1B, a potent proinflammatory cytokine that is also a powerful inhibitor of gastric acid secretion, is up-regulated by *H pylori* infection and is important in initiating and amplifying the inflammatory response to this infection[6]. Genetic analysis from different race's population[6-12] showed that the IL-1B-511 and -31 polymorphisms in the promoter region are associated with the development of gastric cancer. Currently, several different IL-1B genotyping techniques have been described; these include restriction fragment length polymorphisms (RFLP)[7,12-14] analysis and pyrosequencing. Although RFLP is very simple and can be performed without any expensive instrument, post-PCR process and a particular restriction endonuclease are required. Pyrosequencing is a newly developed typing method based on a real-time DNA sequencing[15,16], but an expensive pyrosequencer is needed for the detection.

Up-to-now, various typing techniques based on different principles, e.g., sequence-specific hybridization[17,18], allele-specific extension[19,20] or ligation[21,22], and structure specific cleavage[23,24], have been developed. However, almost all of these methods require purified genome DNA as the

starting material for PCR. The genome DNA extraction is costly and time-consuming. More over, DNA isolation process causes the risk of sample contamination with foreign DNAs. Since a huge number of SNPs should be typed through multiple regions of interest for clarifying the disease-susceptibility SNPs, a simple, inexpensive and labor-saving genotyping method without the DNA extraction procedure is preferable. Here we propose a single-tube genotyping method by combining whole blood PCR (WB-PCR) and tetra-PCR[25,26]. Neither a post-PCR step nor an expensive instrument is required. The type of SNP could be determined by the length of the amplicons from PCR with whole blood or paper-dried blood in a single tube containing four primers: two inner primers specific to each allele and two outer primers defining the length of allele-specific amplicons. To reduce the sample consumption and to increase the typing speed, microchip electrophoresis[27] was used for the detection. As shown in Figure 1, the procedure is very simple, and all of the reagents necessary for PCR as well as blood sample were added in the tube at a time before PCR. Therefore, the aim of the present study is to demonstrate an inexpensive method for typing gastric cancer related SNPs using whole blood or paper-dried blood as starting materials.

MATERIALS AND METHODS

Reagents

Taq DNA polymerase and ethidium bromide were from Promega (Madison, USA). Agarose gel was from Oxoid Limited (Basingstoke Hampshire, England), and dNTP was from Genebase Gene-Tech (Shanghai, China). All solutions were prepared in deionized and sterilized water. DNA 1000 ladder, DNA 1000 Marker15/1500 bp, DNA Dye, and DNA Gel Matrix were purchased from Agilent technologies. The other reagents used in the experiment are analytical pure grade or molecule biology grade. PCR buffer BL-AMP™ was from Bailong Gene Company (Nanjing, China).

Blood samples

Human blood samples were obtained from 40 patients with clinical confirmation and informed consent. Whole-blood samples were venously collected and treated with dipotassium EDTA anticoagulant. The blood sample for condition test was from a volunteer in our laboratory, and directly treated with dipotassium EDTA, sodium citrate, and sodium heparinate respectively. All of the whole blood samples were stored at + 4℃ before use. Paper-dried blood was prepared by dropping a small amount of anticoagulant blood sample on a piece of sterile filter paper, followed by air dry at room temperature, and stored in vacuum desiccator at room temperature before use.

DNA Extraction

Genomic DNA was extracted from blood samples using standard procedures with phenol-chloroform extraction method to separate proteins from nucleic acids[28]. DNA isolation was carried out by suspending whole blood in equal volume of lyses buffer (10 mmol/L Tris-HCl, pH 7.6, 50 mmol/L $MgCl_2$, 320 mmol/L sucrose and 10 g/L Triton-100). After mixing and centrifugation (2 min at micro-centrifuge), the supernatant fluid was removed. This isolation procedure was performed twice. Then the pellet was dissolved with TES buffer (15 mmol/L Tris-HCl, 15 mmol/L EDTA, 15 mmol/L NaCl, 5 mol/L $NaClO_4$, and 100 g/L SDS; pH 8.0), and incubated at 37℃ for 15 min and at 65℃ for 20 min. DNA was extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids remained in the aqueous phase; cellular proteins were separated into the organic phase or lay at the phase interface. After centrifugation, the organic phase was discarded and the aqueous phase was precipitated with absolute ethanol. The pellet was washed with 700 mL/L ethanol, dried in the still air and suspended in Tris-EDTA buffer (10 mmol/L Tris base, 1 mmol/L EDTA; pH 8.0). The DNA was quantified spectrophotometrically at 260 nm, and then stored at 4℃ before use.

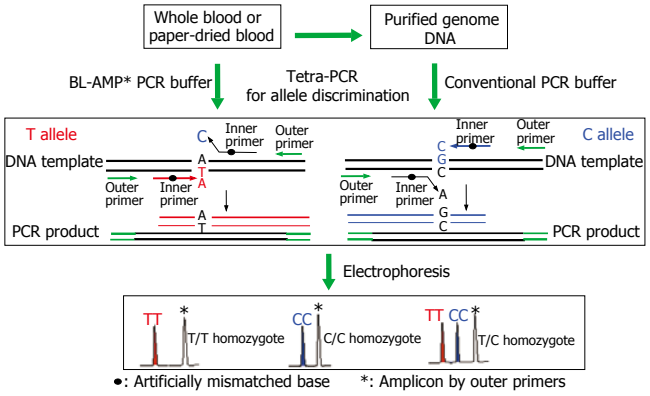


Figure 1 Schematic of a single-tube-genotyping method using tetra-PCR amplification from blood and extracted DNA.

Table 1 Primers for typing polymorphisms IL-1B-31 and IL-1B-511

Polymorphism	Primer sequences
IL-1B-31	P1-1 5′- tcaactgcacaacgattgtca -3′
	P1-2 5′- tcccttagcacctagttgtaa -3′
	S1-1 5′- ttctccctcgctgtttttGta -3′
	S1-2 5′- ctacttctgcttttgaaaCcc -3′
IL-1B-511	P2-1 5′-ggaagggcaaggagtagcaaac-3′
	P2-2 5′-ggcagagctcatctggcattga-3′
	S2-1 5′-ctgcaattgacagagagctGcc-3′
	S2-2 5′-ttgggtgctgttctctgccAca-3′

The capital letters indicated artificial mismatched bases.

PCR primers and target sequences

The nucleotide sequences for the IL-1B gene are available from Genbank (http://www.ncbi.nlm.nih.gov/), and the accession number is AY137079. IL-1B polymorphic sites in the promoter region at position -31 and -511 were genotyped. For typing one SNP, four primers are necessary: two allele-specific primers (S1-1, S1-2 or S2-1, S2-2; Table 1) and two outer primers (P1-1, P1-2 or P2-1, P2-2; Table 1). The sequences of all the primers were listed in the Table 1. A mismatched base was introduced into the third position

upstream from 3' terminal of the allele-specific primer to decrease the probability of spurious extension reaction. Genotypes of IL-1B-31 were determined by a 258-bp PCR fragment (specific to IL-1B-31T) with primers P1-1 and S1-1, and a 162-bp PCR fragment (specific to IL-1B-31C) with primers P1-2 and S1-2. Genotypes of IL-1B-511 were determined by a 356-bp fragment (specific to IL-1B-511C) with primers P2-1 and S2-1, and a 226-bp fragment (specific to IL-1B-511T) with primers P2-2 and S2-2. The software of Primer Premier Version 5.0 (http://www.premierbiosoft.com/) was used to calculate the melting point and to analyze the risk of primer-dimer formation and secondary structures. All the oligonucleotides were synthesized and purified by Invitrogen Biotechnology Co., Ltd (Shanghai, China).

Tetra-primer PCR amplification

Purified DNA template was amplified in a final volume of 15 μL containing 0.2 mmol/L dNTP mixture, 1.67 μmol/L of each of four primers (IL-1B-31), or 2.67 μmol/L of each outer-primer and 0.67 μmol/L of each inner-primer (IL-1B-511), 1 U of Promega *Taq* DNA polymerase, 10 × Buffer, and 1.5 mmol/L $MgCl_2$. Thermocycling was carried out on PTC-225 Peltier Thermal Cycler (MJ RESEARCH, INC.) at an initial denaturation at 96℃ for 3 min, then 35 cycles of 94℃ for 30 s, 52℃ (IL-1B-31) or 58℃ (IL-1B-511) for 1 min, and 72℃ for 1 min, followed by 72℃ for 7 min. Whole blood or paper-dried blood sample was amplified in a 20 μL PCR mixture containing BL-AMP™ buffer instead of the conventional 10 × PCR buffer, 0.2 mmol/L dNTP mixture, 1.5 mmol/L $MgCl_2$, 2 U of Promega *Taq* DNA polymerase, and 1.5 μmol/L of each of four primers (IL-1B-31), or 3 μmol/L of each outer-primer and 0.75 μmol/L of each inner-primer (IL-1B-511). Following the assembly of the PCR mixture, anticoagulant-treated whole blood or filter paper-dried blood was added (commonly 1 μL of blood per 50 μL of PCR mixture) into the bottom of the PCR tube without any agitation. Thermocycling was carried out at an initial denaturation at 96℃ for 3 min, then 40 cycles of 94℃ for 30 s, 52℃ (IL-1B-31) or 58℃ (IL-1B-511) for 1 min, and 72℃ for 1 min, followed by 72℃ for 7 min.

Gel and microchip electrophoresis of PCR products

PCR products were separated by either gel electrophoresis or microchip electrophoresis. The gel electrophoresis was performed on a 20 g/L agarose gel stained with 0.005 g/L EtBr in 1 × TAE buffer at 100 V for 14 min. Gel images were captured on a Bio Imaging System (Syngene, UK). Microchip electrophoresis was carried out on Agilent 2100 bioanalyzer (Agilent technologies, German) according to the manufacture's instructions.

RESULTS

Direct PCR on whole blood and paper-dried blood

To evaluate whether or not target sequences in whole blood or paper-dried blood could be amplified using the "BL-AMP™" buffer, PCR with two sets of primers (P1-1 and P1-2, and P2-1 and P2-2; Table 1), which were used as outer primers for typing IL-1B-31 and -511 polymorphisms, were carried out. Except for PCR buffer, all PCR conditions including *Taq* polymerase, concentration of Mg^{2+} and dNTPs and thermal cycling conditions, are the same as that used in conventional PCR. As shown in Figure 2, the fragments with the expected length of 379 bp and 539 bp were successfully amplified from whole blood with the anticoagulant of dipotassium EDTA, sodium citrate, or sodium heparinate respectively. To check whether paper constitutes suppress PCR or not, a piece of filter paper with 1 mm^2 was added into 50 μL of PCR reaction mixture containing purified genome DNA; and the results showed that "BL-AMP™" buffer could block the PCR inhibition by the chemicals present in filter paper (data not shown). Direct PCR with two sets of primers (P1-1 and P1-2, and P2-1 and P2-2; Table 1) using 0.5, 1, 2 and 4 μL of EDTA-blood dried on filter paper as starting materials were carried out to evaluate the sensitivity (Figure 3); and PCR with 1 μL of fresh blood without an anticoagulant was performed in parallel for the comparison. Successful PCRs were obtained for all samples, and 1 μL of paper-dried blood gave an optimum sensitivity.

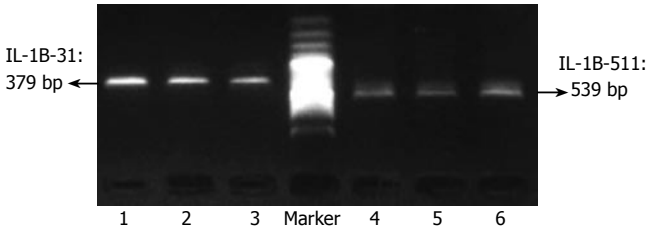


Figure 2 Direct PCR amplification from blood samples with the anticoagulant of dipotassium EDTA (1, 4); Sodium citrate (2, 5); and Sodium heparinate (3, 6).

Genotyping of IL-1B-31 and -511 polymorphisms from various forms of blood samples

By coupling the "BL-AMP™" buffer with tetra-PCR, genotyping of IL-1B-31 and -511 polymorphisms was carried out by using whole blood (with dipotassium EDTA anticoagulant) and dried blood (on a piece of sterile common filter paper) as the starting material respectively. Samples from fifteen different individuals were employed for the evaluation. Amplicons were separated by slab gel electrophoresis that is a simple and inexpensive way in a regular lab. As shown in Figures 4 and 5, it is very easy to get unambiguous SNP types from electrophoretic patterns by using whole-blood samples or paper-dried blood samples as PCR starting materials. As the amplification efficiency of two allele-specific amplicons is different, the concentration ratios of two allele-specific primers were optimized as 8:1 (S1-1:S1-2) to get a relatively equal band-intensities (Figure 6). The bands of 162 bp and 258 bp represent allele C and T respectively. The band of 379 bp was the amplicon by two outer primers P1 and P2; this band was used to be a positive control for checking the sample quality. For a heterozygote sample, both 162- and 258-bp fragments were co-amplified, while either 162- or 258-bp fragment was amplified if the sample was of a homozygote. All of three genotypes were unambiguously determined, indicating that the newly developed method is

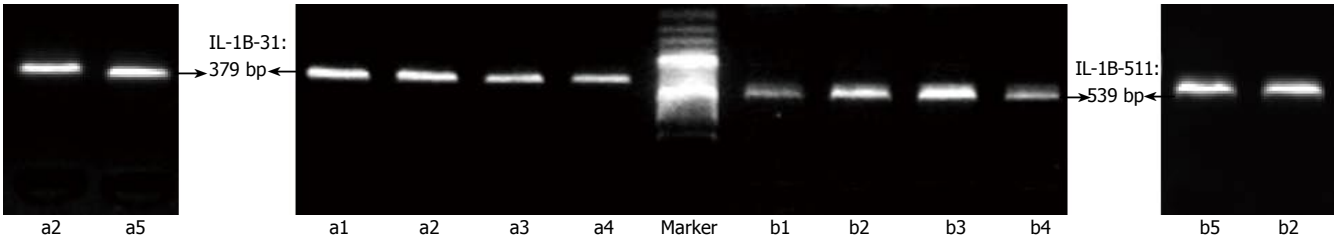


Figure 3 Direct PCR with two sets of primers (P1-1 and P1-2; P2-1 and P2-2) using 0.5 μL (a1, b1), 1 μL (a2, b2), 2 μL (a3, b3) and 4 μL (a4, b4) of EDTA-blood dried on filter paper as starting materials. PCR with 1 μL of fresh blood without an anticoagulant was performed in parallel for the comparison (a5, b5). a1-a5: 379-bp fragment; b1-b5: 539-bp fragment.

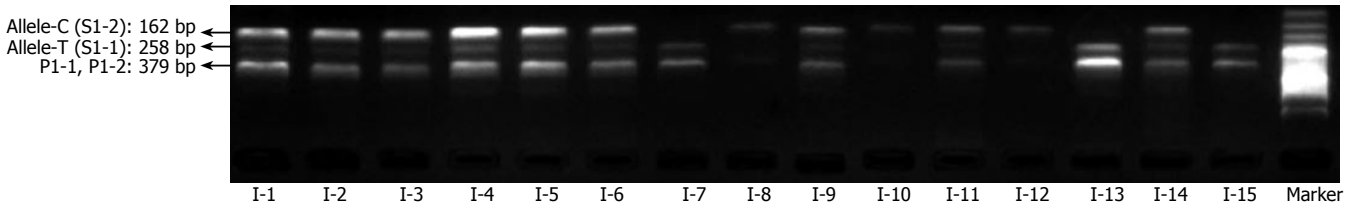


Figure 4 Results of genotyping of IL-1B-31 using whole blood as the starting PCR material.

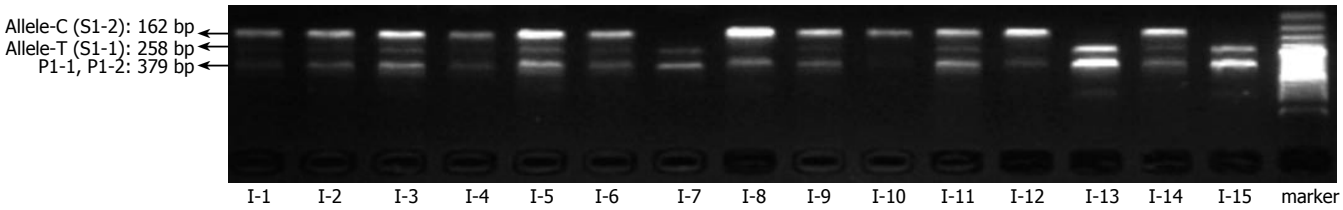


Figure 5 Results of genotyping of IL-1B-31 using paper-dried blood as starting PCR material.

highly specific.

To test the effect of inhibitors in blood or paper tissues on the specificity, genotyping was also performed on the genome DNAs extracted from the same blood samples as those in Figure 4; the results (Figure 7) were in complete agreement with those obtained with whole blood or paper-dried blood samples. Therefore the presence of the inhibitors did not interfere with the specificity of genotyping. By using the same condition, polymorphism of IL-1B-511 was typed directly from fifteen blood samples as shown in Figure 8. The allele-specific PCR fragments are marked by allele-C and allele-T, which correspond to the amplicons with the size of 226 bp and 356 bp respectively. A 539-bp amplicon was from two outer primers (P2-1 and P2-2). The unambiguous typing results of all tested samples were obtained. Also the genotyping results of IL-1B-511 polymorphism from three different starting materials (whole blood, paper-dried blood and purified DNA) are completely consistent (data not shown).

Compared with slab gel electrophoresis, microchip electrophoresis is time-saving, labor-saving, and extremely sensitive. As the separation is in the microchannel, a small amount of DNA sample is enough for getting a reliable result; the typing cost can thus be further decreased. In this report, Agilent 2100 bioanalyzer was employed to analyze the amplified products of the above fifteen blood

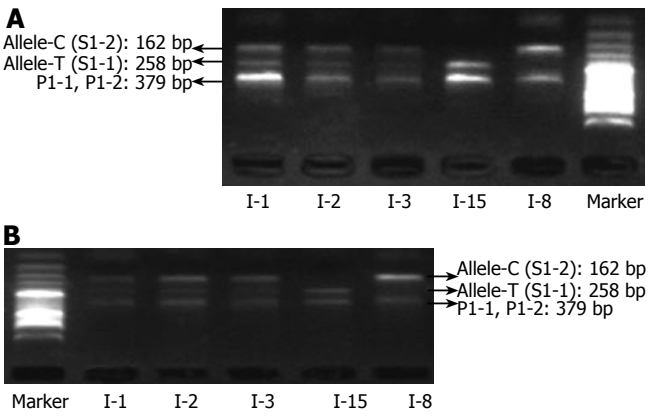


Figure 6 The optimized experiment results of polymorphism IL-1B-31 by changing the concentration ratios of two allele-specific primers from 1:1 to 8:1 (S1-1: S1-2) to get relatively equal band-intensities. **A**: PCR using whole blood as starting PCR materials; **B**: PCR using paper-dried blood as starting PCR materials.

samples. With the DNA markers of different sizes, the length of each amplicon was identified automatically. We found that all blood-based tetra-PCR amplicons gave the expected sizes. Figures 9A and 10A show the typing results of three typical genotypes (two kinds of homozygotes and a heterozygote) of IL-1B-31 and IL-1B-511 respectively.

To evaluate the accuracy of SNP typing, five representative individuals including heterozygotes and

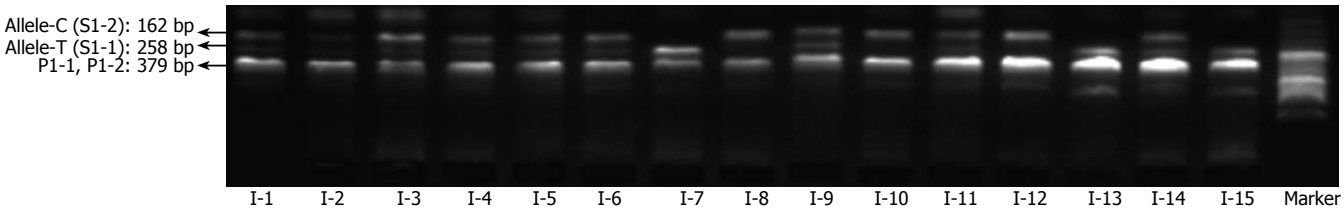


Figure 7 Results of genotyping of IL-1B-31 using extracted genomic DNA as starting PCR materials.

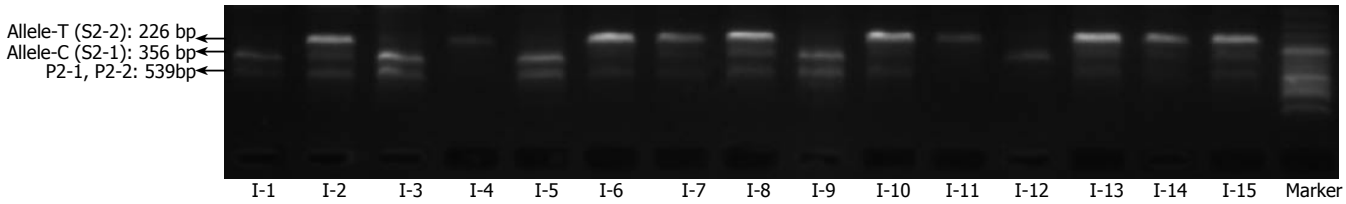


Figure 8 Results of genotyping of IL-1B-511 using whole blood as starting PCR materials.

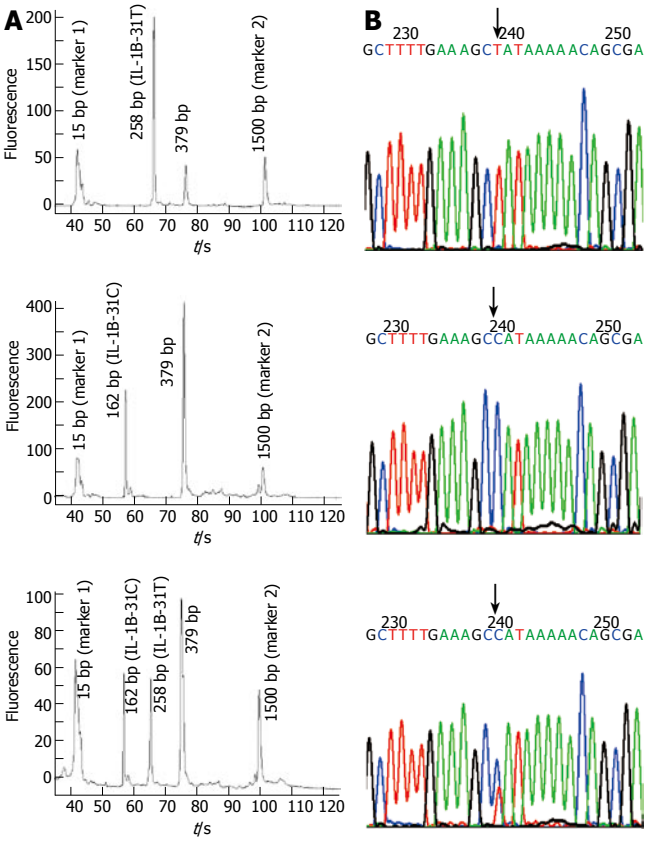


Figure 9 Electrophoresis results of three typical genotypes were obtained, heterozygote (C/T) and both homozygotes (T/T and C/C) of IL-1B-31, by the microchip (**A**) and Sanger's sequencing method (**B**) respectively. The fragment of 379 bp was from two outer primers (P1-1 and P1-2).

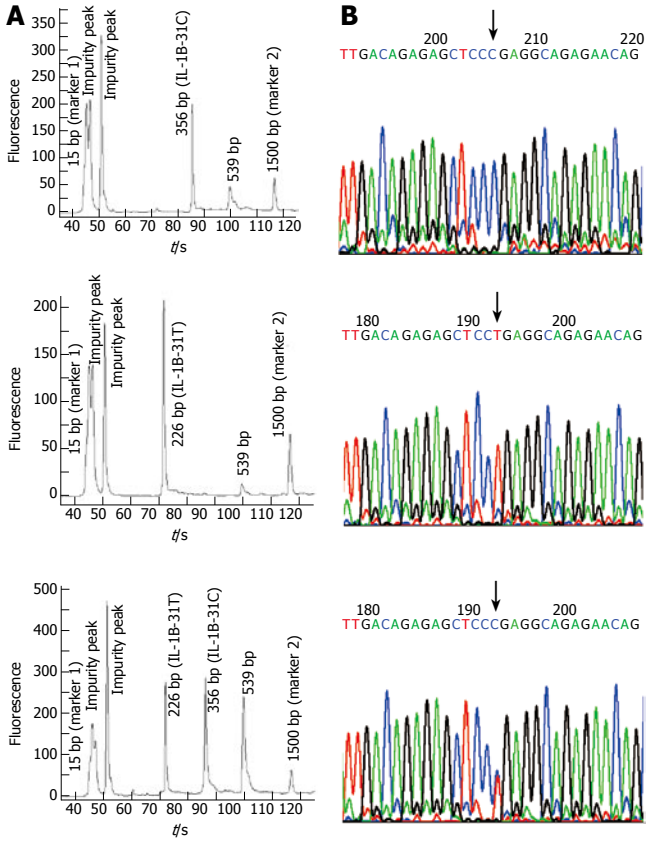


Figure 10 Electrophoresis results of three typical genotypes were obtained, heterozygote (C/T) and both homozygotes (T/T and C/C) of IL-1B-511, by the microchip (**A**) and Sanger's sequencing method (**B**) respectively. The fragment of 539 bp was from two outer primers (P2-1 and P2-2).

both homozygotes were selected and genotyped again by Sanger's sequencing method using purified genomic DNA. The sequencing-based typing results of the five individuals completely coincided with those by the single-tube tetra-PCR. For the comparison, sequencing spectra of the samples used in Figures 9A and 10A were listed as Figure 9B and 10B respectively, indicating that it is very accurate to apply the newly developed single-tube method for genotyping IL-1B-31 and -511 polymorphisms.

To test the robustness of the method, we analyzed three blood samples with typical genotypes for ten times at ten different days. The consistent typing results at both IL-1B-31 and IL-1B-511 polymorphisms were obtained, indicating that the proposed method is reproducible

and reliable. In addition to the blood samples from 15 individuals, we also typed another 25 whole-blood samples by using the proposed single-tube-genotyping method; and unambiguous typing results of both IL-1B-31 and IL-1B-511 polymorphisms were obtained (data not shown).

DISCUSSION

Tetra-PCR is very simple and time-saving[25,26]; but it is not a true single-tube genotyping method as DNA isolation step was still required. Since many substances existing in blood, e.g. polysaccharides, proteins, lipids and haemoglobin, suppress PCR[29], it is very difficult to escape the DNA extraction step and directly use whole blood for PCR amplification. Moreover, anticoagulants, such as EDTA or heparin, which are commonly added to prevent blood samples from the clotting, may interfere with PCR amplification. To effectively suppress the inhibitory substances including the anticoagulant in blood, a novel PCR buffer named "BL-AMP™" was developed.

By coupling tetra-PCR amplification with this buffer, a single-tube-genotyping method using the whole blood and paper-dried blood as the starting material was realized. To type each allele, four primers (two outer primers and two inner primers) were used for amplifying two allele-specific fragments. Two inner primers complementary to sense and non-sense strands respectively were used to discriminate the allele. As one base differing at the 3'-ends of each inner primer is not enough to perfectly switch the allele-specific extension reaction, an artificial mismatch base was introduced into the third base from the 3'-ends. Two outer primers define the length of two allele-specific amplicons. After PCR, amplicons were separated by electrophoresis, e.g., slab gel electrophoresis or microchip electrophoresis. The SNP type is discriminated by the pattern in the electrophoretogram; two bands mean a heterozygote and one band means a homozygote. The length of the band determines the genotype of the homozygote. Unlike Taqman, molecular beacon, or minisequencing, the newly proposed method does not require labeling, and less sample manipulation than RFLP is needed. As the success in avoiding DNA isolation, the genotyping is a true single-tube format by using "BL-AMP™" buffer for PCR.

From the results of our study, it is demonstrated that PCR amplification using "BL-AMP™" buffer is independent of the type of anticoagulant in blood. In terms of transportation and storage of blood samples, it is very convenient to use filter paper as a carrier because no refrigeration is required for paper-dried blood; this is thus very useful to collect blood samples in remote areas. In addition, no significant difference in PCR efficiency between 1-μL fresh blood and dried blood equivalent to 1-μL fresh blood was observed. Compared with conventional PCR using purified DNA, a reddish color in the final PCR products was observed; however, this would not interfere with the detection of PCR products via electrophoresis. Consequently whole blood or paper-dried blood other than purified genome DNA can be employed for the starting materials of PCR.

Although no clean-up process was used after whole-blood PCR, direct microchip electrophoresis of raw PCR products gave a steady baseline. It is straightforward to discriminate genotypes with peaks in the electrophoreic spectra. Based on the peak assignment, genotypes can be accurately determined even if unspecific amplicons occurred in the spectra, so that it becomes easier to design primers for SNP typing by tetra-PCR. On the other hand, it is not necessary to optimize the primer concentration to get an even amplification as the Agilent 2100 bioanalyzer can assign the size to peaks with lower intensities.

In summary, we have successfully proposed a simple, labor-saving, inexpensive and rapid genotyping method that directly uses whole blood or paper-dried-blood as starting materials for SNP typing in a single tube. The successful genotyping of the IL-1B-31 and -511 polymorphisms demonstrated the reliability of the single-tube genotyping using whole blood or paper-dried blood as the starting material. As the DNA extraction step is taken out, the possibility of sample contamination is significantly decreased. By coupling CE-chip for the readout, an automatic and high-throughput genotyping could be achieved. In the post-genome era, a simple and inexpensive method for diagnostic analysis is highly demanded, so we believe that the whole-blood PCR should be very useful for genetic diagnosis in biologic laboratories.

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Increased hepatic expression of insulin-like growth factor-I receptor in chronic hepatitis C

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Abstract

AIM: Although increased insulin-like growth factor-I receptor (IGF-IR) gene expression has been reported in hepatocellular carcinoma, studies assessing IGF-IR in chronic hepatitis C (CHC) and cirrhosis are scarce. We therefore aimed to evaluate IGF-IR and IGF-I mRNA expression in liver from patient with CHC.

METHODS: IGF-IR and IGF-I mRNA content were determined by semi-quantitative RT-PCR and IGF-IR protein expression was determined by immunohistochemistry in hepatic tissue obtained from patients with CHC before (34 patients) and after (10 patients) therapy with interferon-α and ribavirin.

RESULTS: An increase of IGF-IR mRNA content was observed in hepatic tissue obtained from all CHC patients as well as from 6 cadaveric liver donors following orthopic transplantation (an attempt to evaluate normal livers) in comparison to normal liver, while no relevant modifications were detected in IGF-I mRNA content. The immunohistochemical results showed that the raise in IGF-IR mRNA content was related both to ductular reaction and to increased IGF-IR expression in hepatocytes. A decrease in IGF-IR mRNA content was observed in patients who achieved sustained virological response after therapy, suggesting an improvement in hepatic damage.

CONCLUSION: The up-regulation of IGF-IR expression in hepatocytes of patients with CHC could constitute an attempt to stimulate hepatocyte regeneration. Considering that liver is the organ with the highest levels of IGF-I, our finding of increased IGF-IR expression after both acute and chronic hepatic damage highlights the need for additional studies to elucidate the role of IGF-I in liver regeneration.

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Key words: Chronic hepatitis C; Insulin-like growth factor I; Insulin-like growth factor I receptor; Ductular reaction; Hepatocyte regeneration; Ischemia-reperfusion

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INTRODUCTION

There is a close relationship between the endocrine system and the liver, in which processes such as hormonal inactivation, synthesis of hormonal binding proteins, and growth factors take place. The insulin-like growth factor (IGF) system is an attractive target of study, since it may be modified by liver diseases and, reciprocally, it might play a relevant role in the progression of some hepatic diseases. This system is comprised of two ligands, IGF-I and IGF-II, their specific receptors, IGF-IR and IGF-IIR, and the IGF-binding proteins (IGFBPs)[1-4]. IGFs are peptides involved in proliferation, differentiation and inhibition of apoptosis of several cell types[5]. IGF-IR has been shown to be capable of mediating both IGF-I and IGF-II signalling, whereas IGF-IIR (mannose 6-phosphate) is only able to decrease the bioavailability of IGF-II[6].

Circulating IGF-I is synthesized primarily by the liver in response to growth hormone, but most tissues express

IGF-I, which exerts autocrine and paracrine functions[7]. Within the normal adult liver, IGFs are believed to play relatively little effect, since IGF-II expression is down-regulated and IGF-I, although highly expressed, does not exert its actions due to low IGF-IR expression on hepatocytes[8]. Therefore, liver is the organ with the highest levels of IGF-I expression, while it exhibits almost undetectable levels of IGF-IR mRNA. This pattern of expression may be partially explained by down-regulation of IGF-IR promoted by locally produced IGF-I[9]. In contrast to hepatocytes, it is believed that the presence of IGF-IR in non-parenchymal rat liver cells (Kupffer cells, myofibroblasts and hepatic stellate cells [HSC])[10] and in human HSC[11] makes these cell types susceptible to IGFs mitogenic effects.

The role of IGF-I system in hepatic diseases and in liver regeneration remains uncertain. Although an elevation of IGF-IR expression during progression of preneoplastic hepatic foci to hepatocellular carcinoma (HCC) has been reported[12], studies using cultured HCC cells demonstrated a failure of IGF-I to stimulate mitogenesis[10,13,14]. On the other hand, hepatic over-expression of the IGF-II gene has been observed in animal models of hepatocarcino genesis[15,16] as well as in human HCV-induced cirrhosis and HCC[17-19]. Increased IGF-IR gene expression was observed in HCC and in human hepatoma cell lines[8,12,20], however, there are few studies evaluating different chronic hepatitis C (CHC) staging and cirrhosis concerning IGF-IR expression[11,18]. Therefore, the present study was designed to semi-quantify the hepatic expression of IGF-IR and IGF-I in patients with CHC, searching for possible correlations with histopathological lesions in each compartment of hepatic acini.

MATERIALS AND METHODS

Patients and tissue samples

A series of 34 patients with CHC was evaluated at the Clinical Hospital of the University of Sao Paulo Medical School. The diagnosis of CHC was based on a positive serological test for HCV by at least a second-generation enzyme-linked immunoabsorbent assay, positive HCV-RNA by polymerase chain reaction (PCR) assay, and compatible liver biopsy. All patients received combined treatment with interferon-α (3 million U three times a week) and ribavirin (1000-1200 mg/d, accordingly to body weight) for 48 wk, since genotyping was not available. Hepatic tissue fragments obtained before treatment were divided in two samples: one was formalin-fixed paraffin-embedded for histopathological and for immunohistochemical studies; the other was collected in sterile containers and frozen in liquid nitrogen for RNA extraction. Additionally, in 10 patients, liver biopsy was performed within one month after the end of treatment. Among the 34 treated patients, 14 (41.1%) patients reached sustained virological response, 16 (47.05%) patients were non-responders and 4 (11.76%) patients relapsed in the follow-up period. Among the 10 patients histologically evaluated in the post-treatment period, 5 (50%) patients were sustained virological responders and 5 (50%) patients were non-responders.

In order to obtain normal hepatic tissue, hepatic fragments were collected from six cadaveric liver donors (8-12 h of cold ischemia) following orthopic transplantation. The organs obtained from the six cadaveric donors were non-diseased liver transplanted in patients presenting HCV-related end-stage liver disease. The transplants were performed at Experimental Surgery Division, Clinical Hospital, University of Sao Paulo Medical School.

This study was approved by the Ethical Committee of the University of Sao Paulo Medical School and informed consent was obtained from all patients.

Histological and immunohistochemical analyses

Formalin-fixed paraffin-embedded samples were sectioned at a thickness of 3 μm, stained by hematoxylin and eosin (H&E), Masson's trichrome staining and reticulin impregnation, and evaluated by a single pathologist. Histopathological classification was based on criteria defined by the Brazilian Consensus on Chronic Hepatitis[21], which assesses individual histologic variables from zero (normal) to four (maximal degree of lesion): staging of architectural disturbances/fibrosis, necro-inflammatory lesions in portal tract, interface hepatitis and parenchymal lesions. Steatosis was also graded as follows: 0 = no fat deposition in hepatocytes; 1 = up to 25% of hepatocytes; 2 = 25%-50%; 3 = 51%-75%; and 4 = more than 75% hepatocytes presenting fat vacuoles.

Immunohistochemistry for IGF-IR was performed on sections deparaffinized with xylene, dehydrated with graded ethanol, incubated in 3% H_2O_2 to block endogenous peroxidase activity. To improve the staining pattern, antigen retrieval was obtained by heating in 0.5 mol/L EDTA (pH 8.0). The sections were incubated with Protein Block System and Biotin Blocking System (X0909, DAKOCytomation, USA). The primary antibody, mouse monoclonal IGF-IR (α-subunit) Ab-1 (Clone 24-31) (LAB VISION Corporation, Westinghouse, USA) at a dilution of 1:50 was incubated overnight. Signal amplification was achieved by kit LSAB-HRP (Labelled Streptavidin-Biotin) (DAKOCytomation, USA). Chromogen solution included 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., USA). A sample from liver with cholangiocarcinoma was considered as a positive control and a normal liver sample as negative control. The staining was graded according to the estimative of fraction of positive cells of the interlobular bile ducts, lymphocytes from portal tracts and lobular areas, Kupffer cells, hepatocytes, endothelial cells and ducts cells from periportal areas (canals of Hering). Scores were predefined as follows: 0 = without staining; 1 = small amount of immuno-positive liver cells; and 2 = large amount of immuno-positive liver cells.

Semi-quantitative RT-PCR

After tissue pulverization with a dismembrator (B. Braun Biotech International, Melsungen, Germany) at liquid nitrogen temperature, total RNA was extracted using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. After phenol treatment and drying, RNA was dissolved in RNase-free water and RNA concentration was spectrophotometrically determined. RNA quality was checked on agarose gel electrophoresis. The expressions of IGF-

IR and IGF-I in the tissue series were performed by using semi-quantitative RT-PCR. Total RNA (3 μg) was reverse-transcribed into complementary DNA (cDNA) using the SuperScrip™ II Reverse Transcriptase and random primers (Invitrogen Life Technologies) according to manufacturer's recommendations and diluted with ddH_2O to a final volume of 20 μL. The optimal number of PCR cycles was determined for each primer set so that the amplification process was conducted during the exponential phase of amplification. Concomitant amplification of the break-point control region (BCR) gene transcript was used as the internal control to stringently control for any variability in RNA degradation and RT efficiency. Reactions were carried out in a 45 μL final reaction volume containing 3 μL of cDNA template, 20 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.22 mmol/L deoxynucleotide triphosphates (dNTPs), 2.0 U *Taq* DNA polymerase (GE Healthcare-Amersham plc, Buckinghamshire, UK) and specific primers for the target genes and for BCR gene. In order to achieve exponential co-amplification for both IGF-IR and IGF-I mRNA expressions in relation to the endogenous internal control (BCR) at the same number of cycles, sense and antisense primer concentrations were appropriately adjusted between 0.22 and 0.44 μmol/L. PCR amplifications for both genes were performed under the following cycling conditions: initial denaturation at 94℃ for 2 min 30 s, denaturation at 94℃ for 30 s, annealing at specific temperature 57℃ for 1 min and extension at 72℃ for 1 min 30 s, followed by a final extension at 72℃ for 1 min 30 s. The number of cycles to achieve the exponential phase was 40. As a positive control for IGF-IR and IGF-I, human placenta was used. For comparison purpose, normal human liver cDNA was commercially acquired (Human Liver QUICK-Clone cDNA [Clontech Laboratories, Inc., Palo Alto, CA, USA]) and, according to the manufacturer's instructions, cDNA was generated from Poly A+ RNA from samples obtained from two normal human livers. The PCR products were submitted to electrophoresis through a 20 g/L agarose gel containing ethidium bromide and visualized under ultraviolet light. The band intensities were analysed by Molecular Analyst Software (Bio-Rad Laboratories, Hercules, USA). The expression levels of IGF-IR and IGF-I were normalized by the BCR housekeeping gene expression and the ratios of gene expression (IGF-IR or IGF-I/BCR) were calculated in all samples as arbitrary units of optical density (AU). The primers were designed with the primer3_www.cgi v 0.2 program[22] as follows: BCR (377-bp product): 5'-GAG AAG AGG GCG AAC AAG-3' (sense) and 5'-CTC TGC TTA AAT CCA GTG GC-3' (antisense); IGF-IR (230-bp product): 5'-ACC CGG AGT ACT TCA GCG CT-3' (sense) and 5'-CAC AGA AGC TTC GTT GAG AA-3' (antisense); IGF-I (303-bp product): 5'-TCT TGA AGG TGA AGA TGC ACA CCA-3' (sense) and 5'-AGC GAG CTG ACT TGG CAG GCT TGA-3' (antisense).

Statistical analysis

Data were tested for statistical significance using the JMP version 5.1 statistical computer program (SAS Institute Inc. Cary, NC, USA). Since assumptions for a parametric test were not valid (Kolmogorov-Sminov, $P < 0.05$), all data were evaluated by Kruskall-Wallis analysis of variance and the Mann-Whitney *U* test as a multiple comparison method. The Spearman test was used to assess the statistical significance of correlations among the different variables tested. Chi-square and Fisher's exact tests were used to establish statistical relationship between groups regarding histopathological and immunohistochemical findings. *P* values less than 0.05 were considered statistically significant.

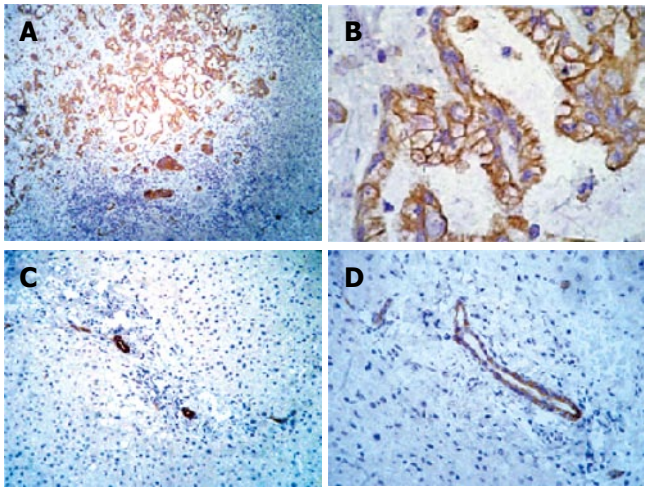


Figure 1 Immunohistochemical analysis of IGF-IR in liver tissue. **A**: Positive control cholangiocarcinoma tissue showing strongly positive staining for IGF-IR in neoplastic ducts (-100 ×); **B**: positive control cholangiocarcinoma tissue showing strongly positive staining for IGF-IR in neoplastic ducts (-200 ×); **C**: normal liver tissue showing IGF-IR immunoreactivity in ductal cells (-100 ×); **D**: normal liver tissue showing IGF-IR immunoreactivity in ductal cells (-200 ×).

RESULTS

Immunohistochemical analysis of IGF-IR

A section of cholangiocarcinoma tissue served as positive control for immunohistochemical analysis of IGF-IR. The positive pattern was observed in cytoplasmic membrane of the neoplastic duct epithelial cells (Figures 1A and 1B). Since positive staining for IGF-IR was detected in ductal cells, they served as internal control, both in normal liver where IGF-IR expression was not detected in hepatocytes (Figures 1C and 1D) and in chronic hepatitis liver samples. In CHC, an expressive increase in IGF-IR immunoreactivity was observed in membrane and in cytoplasm of proliferated bile ducts and in hepatocytes cytoplasm located in all acinar zones (Figures 2A-D). However, nuclear staining was not detected.

Histological and immunohistochemical findings before treatment (samples from 34 patients) and post-treatment (samples from 10 patients) are shown in Figures 3 and 4. Immunohistochemical analyses demonstrated of IGF-IR positivity in hepatocytes in 85% (29/34) samples of CHC before treatment; twenty-three of these 29 samples showed positive immunostaining in hepatocytes located in all regions of the lobe (zones I, II and III of the Rappaport's acinus), while in 6 of these 29 samples revealed positive immunostaining with grade 1 in periportal region, zone 1 (Figure 2E). On contrary, no IGF-IR positivity in

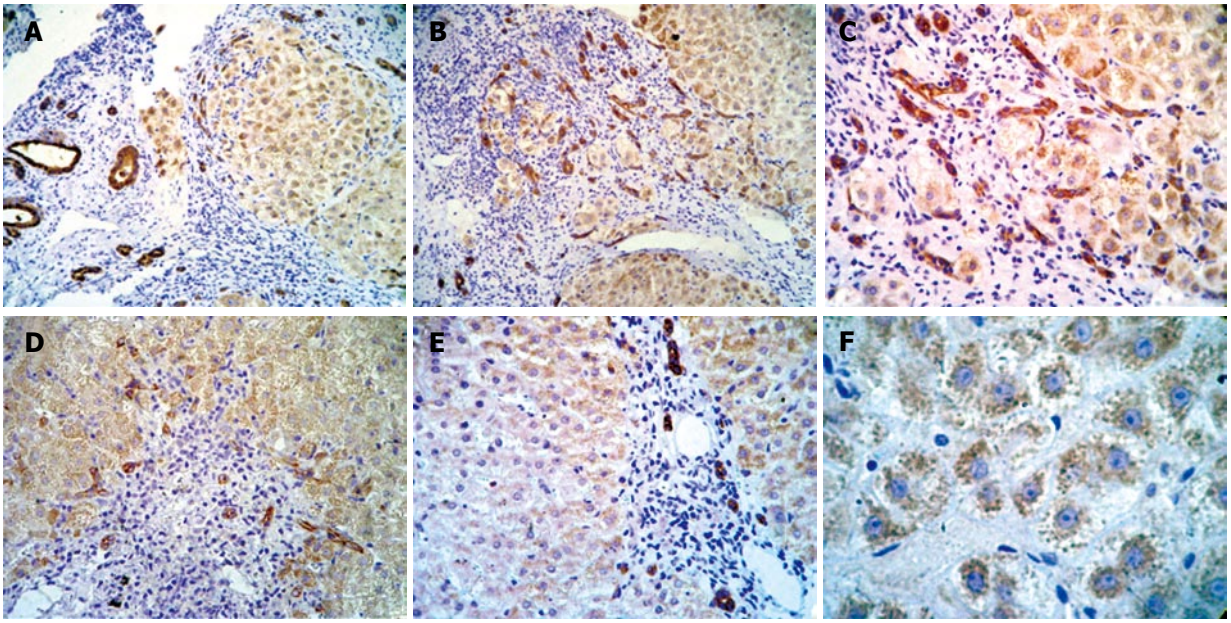


Figure 2 Immunohistochemical analysis of IGF-IR in different samples of liver. **A**: Strong positive staining in cytoplasm of hepatocytes in all acinar zones (-100 ×); **B**: strong positive staining in cytoplasmic membrane and cytoplasm of proliferate bile ducts and in cytoplasm of hepatocytes located in all acinar zones (-100 ×); **C**: strong positive staining in cytoplasmic membrane and cytoplasm of proliferate bile ducts and in cytoplasm of hepatocytes located in all lobular regions (-200 ×); **D**: strong positive staining in cytoplasmic membrane and cytoplasm of proliferate bile ducts and in cytoplasm of hepatocytes located in all acinar zones (-100 ×); **E**: strong positive staining in periportal duct cells (-100x); and **F**: granular pattern of positivity in cytoplasm of hepatocytes in all lobular regions (-400 ×).

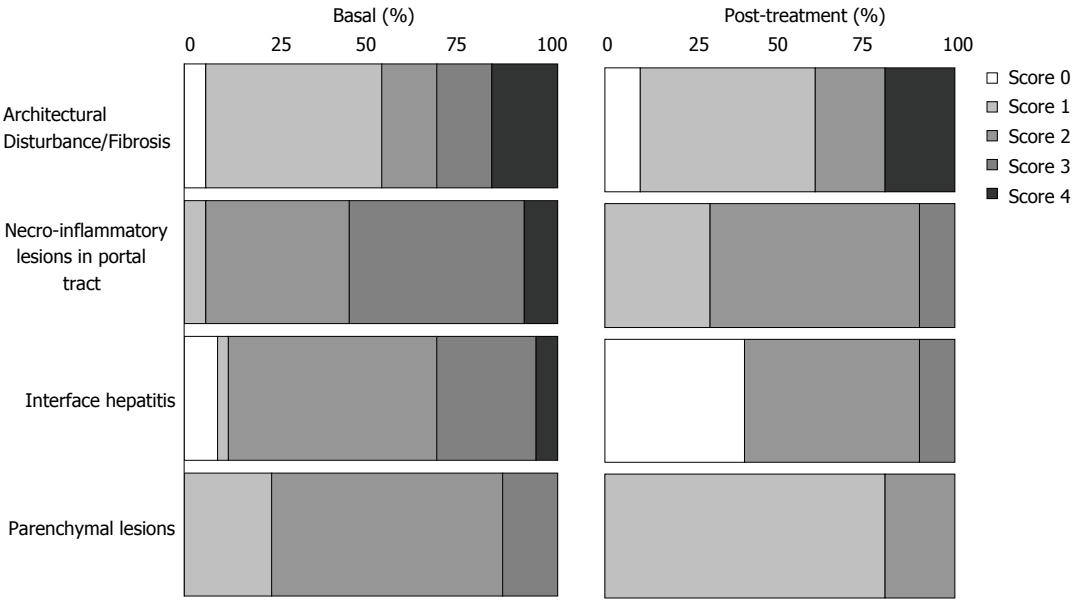


Figure 3 Percentage of score distribution obtained by histological analyses in liver biopsies from pre- (34 cases) and post-treatment (10 cases, 5 sustained virological responders and 5 non-responders) according to Brazilian Consensus on Chronic Hepatitis[21].

hepatocytes was observed in 15% (5/34) samples of CHC before treatment.

Among the patients with fibrosis of degree 0, 1 and 2, 13% (3/23) did not show positivity in hepatocytes, 70% (16/23) showed weak staining and 17% (4/23) showed intense staining. Within the group with fibrosis of degree 3 and 4, 9% (1/11) did not show any staining, 55% (6/11) showed weak staining and 36% (4/11) showed intense staining. In some cases, especially in cirrhotic livers, a granular pattern of positivity in hepatocytes cytoplasm from all lobular regions was visualized (Figure 2F).

All of the pre-treatment samples (34/34) showed IGF-IR positivity in bile ducts, with strong positivity (degree 2) in 91% (31/34) of the positive cases. Twenty-eight of 34 (82%) samples showed IGF-IR positivity in periportal duct cells (canals of Hering), with weak positivity (degree 1) in 25 of 28 samples and strong positivity (degree 2) in 3 of 28 samples.

Only 50% (17/34) and 15% (5/34) of the samples showed positivity in portal lymphocytes and Kupffer cells, respectively, with a weak (degree 1) positivity pattern. No staining in lobular lymphocytes and in endothelial cells was observed.

Histopathological and immunohistochemical variables

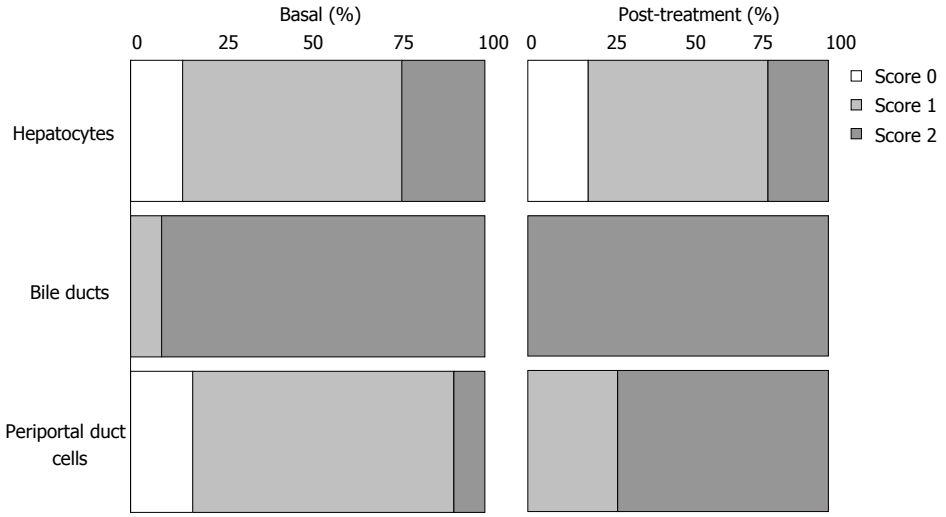


Figure 4 Percentage of score distribution obtained by IGF-IR immunohistochemical analyses in liver biopsies from pre- (34 cases) and post-treatment (10 cases, 5 sustained virological responders and 5 non-responders).

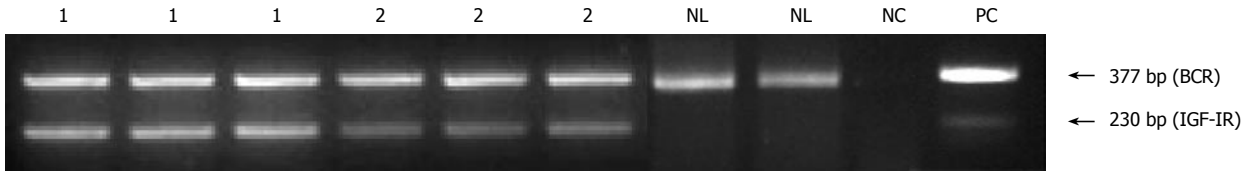


Figure 5 Semi-quantitative RT-PCR analysis of IGF-IR mRNA in hepatic fragments obtained from 2 patients with chronic hepatitis C (lanes 1 and 2) and normal human liver (NL). The experiments for the patients with CHC were carried out in triplicates and for normal human liver (NL) in duplicates. Human placenta was used as a positive control (PC) and no DNA template as negative control (NC).

did not show any statistically significant correlation. There were no statistically significant differences on IGF-IR immunohistochemical pattern in hepatic tissues from patients with and without sustained virological response between pre- and post-treatment periods (Figure 4).

mRNA expression of IGF-IR and IGF-I

An increased IGF-IR mRNA content was detected in hepatic tissue of all 34 patients with CHC before treatment as compared with normal liver (Clontech Laboratories) which did not express IGF-IR mRNA (Figure 5). There were no relevant modifications in IGF-I mRNA content in hepatic tissue from patients with CHC as compared with normal liver, however, since there was only one sample of normal hepatic tissue, it was not possible to perform a statistical evaluation (data not shown).

IGF-IR and IGF-I mRNA contents were evaluated in hepatic tissue obtained within one month of the end of treatment in ten patients. There was a statistically significant decrease ($P = 0.05$) in mean IGF-IR mRNA content in hepatic tissues from patients who achieved sustained virological response in comparison to non-responders (Figure 6), while there was no statistically significant difference in IGF-I mRNA content in hepatic tissues from patients with and without sustained virological response between pre- and post-treatment periods (Figure 7). Furthermore, no statistically significant correlations were observed between IGF-IR and IGF-I mRNA contents and histopathological or immunohistochemical variables.

In an attempt to better characterize the expression pattern of these two genes in normal hepatic tissues, IGF-IR and IGF-I mRNA contents were evaluated in hepatic fragments from six cadaveric liver donors (8-12 h of cold ischemia) following orthopic transplantation. Interestingly, despite being considered as normal livers by histopathological examination, all samples presented an increased IGF-IR mRNA content (Figure 8), regardless the degree of ischemia-reperfusion injury, which ranged from 1 to 3, accordingly to Gayotto & Leitão classification[23]. In addition, there were no relevant modifications in IGF-I mRNA content in hepatic tissue from liver donors following orthopic transplantation in comparison to normal liver (data not shown).

DISCUSSION

Although almost all tissues and cells express IGF-IR during embryogenesis, in the normal adult liver, IGF-IR mRNA is practically undetectable[5] and very low IGF-I binding has been shown in cultured hepatocytes[8], possibly due to IGF-IR down-regulation by the locally produced IGF-I. However, hepatic non-parenchymal cells, such as Kupffer cells, endothelial cells and HSC, express IGF-IR and are susceptible to their mitogenic effects[11,24,25].

In the current investigation, we demonstrated an increase of IGF-IR mRNA content in hepatic tissue from patients with different CHC staging. The immunohistochemical findings suggested this augmented mRNA content might be secondary to ductular proliferation, since these epithelial cells expressed IGF-IR, and to increased IGF-IR expression in hepatocytes. Although IGF-IR im-

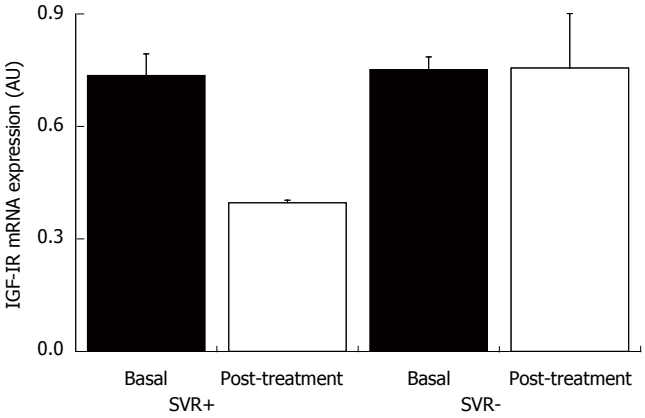


Figure 6 IGF-IR mRNA content in liver tissue from patients with chronic hepatitis C with and without sustained virological response, and pre- and post-treatment with INF-α and ribavirin. SVR+: With sustained virological response (*n* = 5); SVR-: without sustained virological response (*n* = 5).

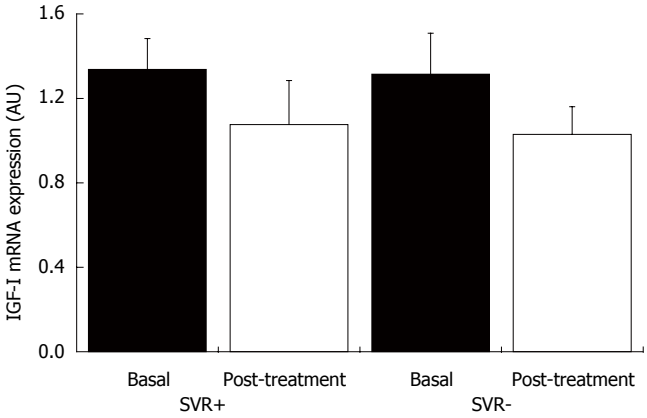


Figure 7 IGF-I mRNA content in liver tissue from patients with chronic hepatitis C with and without sustained virological response, and pre- and post-treatment with INF-α and ribavirin. SVR+: With sustained virological response (*n* = 5); SVR-: without sustained virological response (*n* = 5).

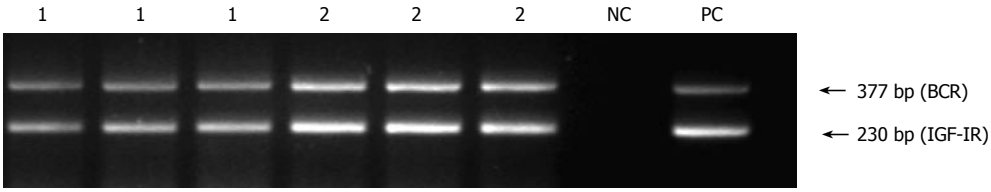


Figure 8 Preservation-reperfusion injury: Representative results of semi- quantitative RT-PCR analysis of IGF-IR mRNA in 2 hepatic fragments from cadaveric liver donor following orthopic transplantation (lanes 1 and 2). The experiment was carried out in triplicates. Human placenta was used as a positive control (PC) and no DNA template as negative control (NC).

munoreactivity was low in Kupffer cells and in portal lymphocytes, we could not rule out that the expression of this receptor in these cell types has also contributed to increase the level of IGF-IR mRNA observed in all cases.

The proliferative response to several types of liver injury in human liver is characterized by an increase in bile duct–like structures, termed "ductular reaction"[26,27]. Ductular reaction located at the limiting plate takes place in hepatitis C[28], where a significant correlation between the extent of ductular proliferation and the severity of inflammation was observed[29]. Eleazar *et al*[30] have also demonstrated a positive correlation between ductular reaction and disease activity in chronic hepatitis, including CHC. Although in the current study, immunohistochemical staining for specific epithelial cell markers such as cytokeratin 19 has not been performed, IGF-IR immunostaining and H&E analysis have clearly shown ductular reaction. Previous findings showing an increase in the number of cells that originate newly proliferated ductal cells, the hepatic progenitor cells ("oval cells"), as hepatitis C severity progresses[31] as well as the recent report of a strong correlation between portal fibrosis and periportal ductular reaction with progenitor cells expansion in CHC[32] illustrate the need for further studies addressing the mechanisms underlying progenitor cells activation and liver remodelling in CHC.

The up-regulation of IGF-IR expression in hepatocytes of patients with CHC could constitute an attempt to stimulate hepatocyte regeneration in response to liver damage. The role of IGF-IR in liver regeneration following subtotal hepatectomy has been demonstrated in animal models. Caro *et al*[8] showed a significant increase in IGF-I binding in freshly isolated hepatocytes from regenerating adult rat liver. In addition, Santos *et al*[33] demonstrated both a transient increase in IGF-I binding to liver membranes and an increase on IGF-IR mRNA concentrations. Up-regulation of IGF-IR as well as IGF-I was also observed in hepatocytes of rats chronically treated with carbon tetrachloride (CCl_4) and immunoreactivity increased proportionally to fibrosis development, which might be a compensatory reaction to the continuous loss of hepatocytes[34]. However, the role of IGF-I system in the injured liver has not been completely understood. While experiments in cultured HSC support the participation of IGF-I in HSC transformation toward a fibrogenic phenotype, promoting HSC proliferation[25,35] and collagen synthesis[11], targeted over-expression of IGF-I in activated HSCs attenuates fibrogenesis and accelerates liver regeneration after acute CCl_4-induced injury. These effects appear to be mediated in part by up-regulation of HGF and down-regulation of TGF-β1, suggesting that IGF-I can modulate the cytokine response to liver injury, facilitating regeneration and reducing fibrosis[36]. Based on our finding of increased IGF-IR expression even after an acute challenge such as ischemia-reperfusion injury, we question if a direct effect of HSC-derived IGF-I on hepatocytes expressing IGF-IR might contribute to the accelerated hepatic regeneration observed in this model. Additionally, Desbois-Mouthon *et al*[37] have recently reported that hepatocyte proliferation

during liver regeneration is impaired in liver-specific IGF-IR knockout mice as evidence that IGF-IR contributes to liver regeneration.

In the liver, it has been shown that ischemia-reperfusion injury occurs as biphasic pattern, with an initial acute phase characterized by hepatocellular damage at 3-6 h and a sub-acute phase characterized by massive neutrophil infiltration at 18-24 h[38]. The ischemia-reperfusion injury also induces the activation of intracellular pathways clearly involved in the hepatocellular regenerative response[39], such as expression of tumor necrosis factor α (TNF-α)[40,41] and IL-6-associated signalling pathways[42], in an attempt to replace cells lost to ischemic and immunologic damage[43]. To our knowledge, augmented IGF-IR expression has never been reported in animal or human livers submitted to ischemia-reperfusion injury, while it was described in a murine model of renal ischemia-reperfusion injury[44]. Considering that liver is the organ with the highest levels of IGF-I expression, our finding of increased IGF-IR expression in an acute model of hepatic damage such as ischemia-reperfusion injury as well as in the condition of chronic hepatitis C highlights the need for additional studies to elucidate the role of IGF-I in the complex events of liver regeneration.

Regarding IGF-IR expression in the post-treatment period, although few patients were available for this analysis, the decrease in IGF-IR mRNA content observed in the patients who achieved sustained virological response suggests an improvement in hepatic injury without returning to the expression pattern observed in normal liver, which exhibits undetectable levels of IGF-IR mRNA. We hypothesized that the change on IGF-IR mRNA content in the absence of modifications on the immunostaining pattern, as well as the positivity of IGF-IR mRNA in all studied samples even in the absence of IGF-IR immunostaining may be explained by the higher sensibility of RT-PCR in comparison to immunohistochemical analysis. It remains to be clarified if, as observed for histopathological changes, longer periods following sustained virological response are require for detecting additional reduction in IGF-IR mRNA content.

Because of the important role of the IGF system in mediating not only normal growth and tissue repair but also carcinogenesis, the expression and function of all components of this system must be strictly controlled. Although we speculate that up-regulation of IGF-IR might participate in hepatocyte regeneration, the suggestion that HCV replication might mediate expression of the IGF-IR ligand IGF-Ⅱ in cirrhotic livers[45] rise concerns that these two simultaneous changes could act as possible factors for HCV-associated hepatocarcinogenesis in cases of advanced hepatic lesion, since an autocrine and/or paracrine activation of the IGF-Ⅱ/IGF-IR axis has been implicated in the development of HCC[46]. This hypothesis is corroborated by the finding that hepatitis B virus (HBV) X protein (HBx), which participates in the process of HBV-associated liver carcinogenesis, activates the IGF-IR gene expression[47,48]. It remains to be elucidated if a direct activation of IGF-IR gene by HCV could contribute to the increased IGF-IR expression in hepatocytes in CHC.

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Defensive nature of *Sargassum polycystum* (Brown alga) against acetaminophen-induced toxic hepatitis in rats: Role of drug metabolizing microsomal enzyme system, tumor necrosis factor-α and fate of liver cell structural integrity

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Abstract

AIM: To assess the defensive nature of *Sargassum polycystum* (*S. polycystum*) (Brown alga) against acetaminophen (AAP)-induced changes in drug metabolizing microsomal enzyme system, tumor necrosis factor (TNF-α) and fine structural features of the liver during toxic hepatitis in rats.

METHODS: Male albino Wistar strain rats used for the study were randomly categorized into 4 groups. Group I consisted of normal control rats fed with standard diet. Group II rats were administered with acetaminophen (800 mg/kg body weight, intraperitoneally). Group III rats were pre-treated with *S. polycystum* extract alone. Group IV rats were orally pre-treated with *S. polycystum* extract (200 mg/kg body weight for 21 d) prior to acetaminophen induction (800 mg/kg body weight, intraperitoneally). Serum separated and liver was excised and microsomal fraction was isolated for assaying cytochrome P450, NADPH Cyt P450 reductase and b_5. Serum TNF-α was detected using ELISA. Fine structural features of liver were examined by transmission electron microscopy.

RESULTS: Rats intoxicated with acetaminophen showed considerable impairment in the activities of drug metabolizing microsomal enzymes, such as cytochrome P450, NADPH Cyt P450 reductase and b_5 when compared with the control rats. The rats intoxicated with acetaminophen also significantly triggered serum TNF-α when compared with the control rats. These severe alterations in the drug metabolizing enzymes were appreciably prevented in the rats pretreated with *S. polycystum*. The rats pretreated with *S. polycystum* showed considerable inhibition in the elevation of TNF-α compared to the rats intoxicated with acetaminophen. The electron microscopic observation showed considerable loss of structural integrity of the endoplasmic reticulum, lipid infiltration and ballooning of mitochondria in the acetaminophen-intoxicated rats, whereas the rats treated with *S. polycystum* showed considerable protection against acetaminophen-induced alterations in structural integrity.

CONCLUSION: These observations suggest that the animals treated with *S. polycystum* extract may have the ability to protect the drug metabolizing enzyme system and mitochondrial functional status from free radical attack, thereby showing its defense mechanism in protecting hepatic cells from acetaminophen toxic metabolite N-acetyl-para-benzoquinone-imine (NAPQI).

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Key words: *Sargassum polycystum*; Acetaminophen; Toxic hepatitis; NAPQI; Free radical

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INTRODUCTION

Our modern lifestyle has drastically changed the chemistry of the environment in which we live. There is ample documentation of increased toxicity in our food, air and water. In addition, chronic illnesses often are connected to the production of toxic chemicals that inflame and cause oxidative stress. Moreover, many medications are metabolized in the liver, and in effect, can function as toxins. Unfortunately, many of us promote an increase of our own toxin levels with excessive alcohol intake, smoking and over counter drug use[1]. Drug detoxification is an important function of the liver. It is a complex

process that occurs in the endoplasmic reticulum of the hepatocyte, which involves several phases. Its main role in detoxification involves neutralizing harmful compounds, such as drugs, pesticides, hormones and bacterial toxins from the intestines.

The seaweeds are considered the most nutritious plants on earth. Their nutritive values greatly exceed those found in other food sources that humans can readily utilize. From the time immemorial the macroscopic marine algae have been closely associated with human life and are being commonly used in numerous ways as a source of food/feed and medicine. In folk medicine, seaweeds have been used for a variety of remedial purposes, such as in eczema, gallstone, renal trouble, scabies, psoriasis, asthma, arteriosclerosis, heart disease, ulcers and cancer. The cell walls of marine algae characteristically contain polysaccharides, which are not found in land plants and which may have specific functions in ionic regulation and wide spectrum of pharmacological importance. Sulphated polysaccharide (e.g. fucoidan) is structurally unique that is found only in the cell walls of several types of brown seaweed. Brown algae substantially differ from algae of other divisions and terrestrial plants in their composition[2,3]. A perusal of literature study has shown that the brown algae contain essential minerals, vitamins, free amino acids, mannitol, glucitols, sulphated polysaccharides and phlorotannins. These compounds in brown algae are found to have wide spectrum of biological properties[4-7].

Based on the previous studies regarding free radical scavenging property of *Sargassum polycystum* (*S. polycystum*) extract against acetaminophen-induced toxic hepatitis in rats, the present study was attempted to assess the defensive nature of *S. polycystum* (Brown alga) extract against acetaminophen-induced alterations in drug metabolizing microsomal enzyme system, TNF-α during toxic hepatitis and cellular structural integrity during toxic hepatitis.

MATERIALS AND METHODS

Sample collection and extraction

S. polycystum was collected from Gulf of Mannar, Rameswaram, India. The species authentication was done by Prof. V. Krishnamurthy (Krishnamurthy Institute of Algology, Chennai, India). The seaweed sample was washed in seawater and then fresh water to remove the epiphytes and other contamination. The coarsely powdered seaweed material was extracted with ethanol in cold for a period of 72 h with occasional shaking. The crude extract was filtered, concentrated on a water bath, and then dried in vacuum. The resulting dried powder was used for the animal experimentation. The extract was subjected to thin layer chromatography (TLC) and phytochemical analysis, which showed positive result for the presence of terpenoids and flavonoids[8].

Animals

Male Wistar strain albino rats, weighing about 100-130, were procured from Fredrick Institute for Plant Protection and Toxicology, Padappai, Chennai, India. The animals were housed in cages under proper environmental conditions and fed with a commercial pelleted diet (M/s Hindustan Foods Ltd, Bangalore, India). The animals had free access to water throughout the experimental period.

Experimental protocol

The experimental animals were divided into four groups, each group comprising six animals. Group I consisted of normal control rats fed with standard diet. Group II rats were intoxicated with acetaminophen (800 mg/kg body weight, intraperitoneally in saline solution in a boiling water bath and used after cooling at 37℃). Group III rats were orally pre-treated with *S. polycystum* extract alone (200 mg/kg body weight for 21 d). Group IV rats were orally pre-treated with *S. polycystum* extract (200 mg/kg body weight for 21 d) prior to acetaminophen induction (800 mg/kg body weight, intraperitoneally).

At the end of the experimental period the animals were deprived of standard diet for 20 h and then anesthetized with diethyl ether, followed by cervical decapitation. Blood collected without anticoagulant for serum. The liver was excised and immersed in ice-cold 0.01 mol/L tris (hydroxymethyl) aminomethane (Tris)-1.15 mol/L KCL buffer (pH 7.4). After rinsing twice in buffer, the liver was weighed and homogenized with a Teflon potter-Elvehjem homogenizer in ice-cold measured volumes of Tris-KCL buffer. The homogenate was then centrifuged at 9000 g for 15 min at 0℃. The microsomal pellet was then layered with 1 mL of Tris-KCl buffer and recentrifuged for an hour at 105 000 g at 0℃.

Microsomal enzyme profile

The microsomal pellets were layered and stored frozen prior to performing enzyme assays. Cytochrome p450 and b_5 assay was performed as previously described by Omura and Sato[9]. NADPH-cytochrome p450 reductase assay performed as previously described by Phillips and Langdon[10].

Tumor necrosis factor-α (TNF-α)

One hundred microgram of the protein sample in carbonate buffer (pH 9.4) was coated on the 96-well polyvinyl chloride ELISA plates and kept overnight at 4℃. The coated wells were washed twice with phosphate-buffered saline (pH 7.4) containing 0.5 mL/L Tween 20, blocked with 10 g/L bovine serum albumin (BSA) in phosphate-buffered saline (100 μL/well) and incubated at 37℃ for 1 h. The coated wells were washed thrice with phosphate-buffered saline containing 0.5 mL/L Tween 20. Then 50 μL of anti-TNF-α was added and incubated at 37℃ for 1 h or overnight at 4℃. After incubation, the ELISA plates were washed as above and 50 μL of anti-mouse goat anti-rabbit-IgG-HRP (1:5000) diluted with phosphate-buffered saline was added and incubated for 1 h. The wells were washed with PBS-Tween 20 thrice and with PBS thrice. Fifty microliter of substrate solution (1 μL of H_2O_2, 0.5 mg OPD in 1 mL citrate-phosphate buffer, pH 5.0) was added. Fifty microliter of 1.5 mol/L H_2SO_4 was added after 30 min to stop the reaction. The intensity of the color was recorded on an ELISA reader at 490 nm[11].

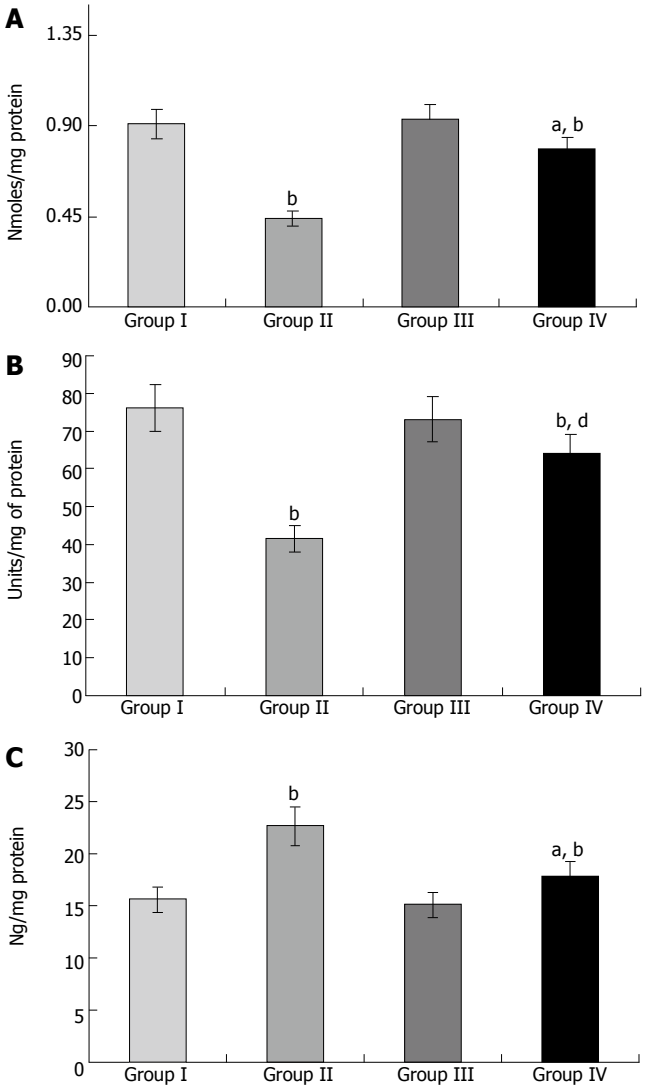


Figure 1 Various effects of *S. polycystum* against acetaminophen-induced toxic hepatitis in rats. **A**: Effect of *S. polycystum* on cytochrome P450 during acetaminophen-induced toxic hepatitis; **B**: Efficacy of *S. polycystum* on cytochrome b_5 against acetaminophen-induced toxic hepatitis; **C**: Effect of *S. polycystum* on the status of microsomal cytochrome P450 reductase. Values are expressed as mean ± SD for six rats. $^{b}P < 0.01$ *vs* group I, $^{d}P < 0.001$ *vs* group II, $^{a}P < 0.05$ *vs* group I.

Transmission electron microscopy

A portion of the liver tissue was instantaneously immersed in 25 g/L of glutaraldehyde solution, buffered with 0.1 mol/L sodium cacodylate (pH 7.4). The specimen was then placed in the buffer fixative medium, followed by washing with sodium cacodylate and fixation in 20 g/L osmium tetroxide buffered with 0.1 mol/L sodium cacodylate. After dehydration in a graded series of alcohol and propylene oxide, the tissues were transferred to the propylene oxide: ethanol mixture (1:1) and embedded in resin. The specimens were mounted on epoxy resin blocks and left in the oven at 65℃ for 72 h. Thin sections were cut with an ultramicrotome, stained with uranyl acetate and lead citrate, and then examined under an EM-9A electron microscope.

Statistical analysis

Values were expressed as mean ± SD for six rats in each group and significance of the differences between mean values were determined by one-way analysis of variance (ANOVA), followed by the Duncan test for multiple comparison. A *P* value less than 0.05 was considered statistically significant.

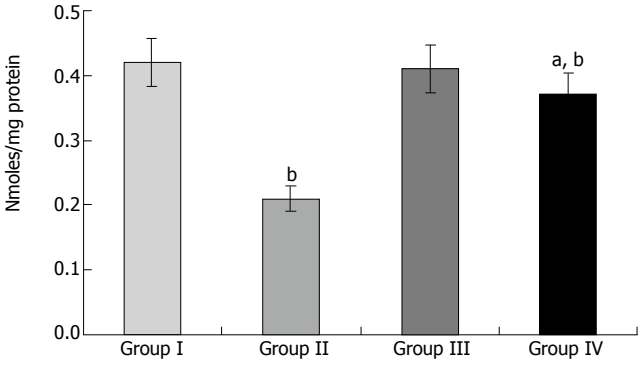


Figure 2 Effect of *S. polycystum* on TNF-α in serum of control and experimental group of rats. Values are expressed as mean ± SD for six rats. $^{a}P < 0.05$ *vs* group I, $^{b}P < 0.01$ *vs* group I, $^{d}P < 0.001$ *vs* group II.

RESULTS

Effect of S. polycystum on microsomal enzymes and TNF-α

Figure 1 shows the activities of microsomal enzymes in the control and experimental group of rats. The group II rats intoxicated with acetaminophen showed considerable impairment in the activities of drug metabolizing microsomal enzymes, such as cytochrome P450, NADPH Cyt P450 reductase and b_5 when compared with group I control rats. Figure 2 shows the serum TNF-α of control and experimental rats. The group II rats intoxicated with acetaminophen also significantly triggered serum TNF-α when compared with group I control rats. However, these severe alterations in the drug metabolizing enzymes were appreciably prevented in group IV rats pretreated with *S. polycystum* when compared with group II intoxicated rats. A considerable inhibition in the elevation of TNF-α was observed in the rats pretreated with *S. polycystum* compared to group II rats intoxicated with acetaminophen.

Effect of S. polycystum on liver cell structural integrity

Figure 3A shows the hepatic transmission electron microscope of the control rat liver. Figure 3B, Figure 3C and Figure 3D show acetaminophen-induced alterations in the structural integrity and lipid membrane of group II rats when compared with group I control animals. Group IV rats pretreated with S. polycystum showed considerable protection against acetaminophen-induced alterations in structural integrity when compared with toxicant group (Figure 3E, Figure 3F, Figure 3G, Figure 3H).

DISCUSSION

Puntarulo and Cederbaum[12] have found a close parallelism between formation of malondialdehyde and loss of microsomal enzyme activities. Cyt P450 and NADPH cytochrome P450 reductase are not rapidly

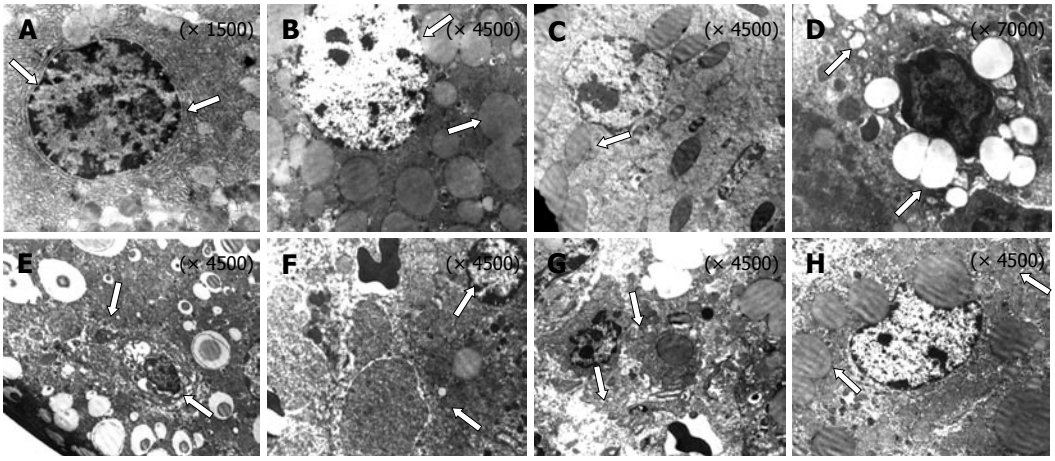


Figure 3 Structural features of liver examined by transmission electron microscopy. **A**: Normal cellular structure of rat liver without any destruction; **B**, **C** and **D**: Effect of acetaminophen on fine ultrastructural changes during toxic hepatitis in rats; **E**, **F**, **G** and **H**: Effect of *S. polycystum* against acetaminophen-induced alterations in structural integrity during toxic hepatitis.

organized in the microsomal membrane and they possess lateral mobility, which largely depends on fluidity of the membrane[13]. Peroxidation of membrane lipids has been shown to repress the membrane fluidity[14]. Therefore, the observed impairment in the activities of xenobiotic metabolizing enzymes in acetaminophen-intoxicated rats could account for the alteration in membrane fluidity.

In the liver, exogenous and endogenous compounds are metabolized by the cytochrome P450-dependent monoxygenase system. The enzymes involved in this process are located in the endoplasmic reticulum of the liver, and their activities are dependent on many environmental factors[15,16]. When considering biotransformation processes, it is important to analyze them together with other cellular processes taking place in the hepatocyte. Impairment of these processes may disturb cellular nutritional status, thus affecting the detoxication reactions[17-19]. This enzyme system, therefore, provides our primary defense against xenobiotics and is a major determinant in the therapeutic efficacy of pharmacological agents[20].

The catalytic cycle of cytochrome P450 (Cyt p450) is complex and involves two single electron transfer process. Electrons are relayed from NADPH to Cyt P450 via the flavoprotein called NADPH Cyt P450 reductase[21]. Cyt b_5 is a small hemoprotein that influences the rate of some Cyt P450 catalyzed reactions[22]. The altered levels of Cyt P450 and Cyt b_5 during acetaminophen administration have also been attributed to the increase in the degradation of heme by acetaminophen-induced heme oxygenase activity. Acetaminophen also affects thiol groups in cytochrome apoproteins and thus prevents heme incorporation[23]. The activity of NADPH Cyt P450 reductase depends upon the concentration of its substrate Cyt P450. Low availability of Cyt P450 might have been responsible for the reduction in the activity of NADPH Cyt P450 reductase. It has also been suggested that, once exposed acutely to xenobiotics such as acetaminophen, hepatic cytochrome P450 can undergo oxidative destruction through its active intermediates and can induce carbon monoxide (CO) overproduction mediated by hemeoxygenase 2 (HO-2), even when the inducible enzyme is not over-expressed[24,25].

The rats pretreated with *S. polycystum* extract showed appreciable prevention in the acute impairment of microsomal enzyme activities when compared with acetaminophen-intoxicated rats. Thus, it is evident in this study that near normal activity of NADPH Cyt P450 reductase indirectly lends support to the hypothesis that NADPH Cyt P450 reductase may be the locus within the cytochrome P450 electron transport chain, which is most susceptible to peroxidative attack by free radicals[26]. A hypothesis states that bioactive compounds that are able to optimize the microsomal enzyme function can provide protection against the hepatotoxicity only when they are given before the metabolic activation of the hepatotoxin and fail to afford any protection after the generation of reactive metabolites[27]. These observations contemplate that the seaweed extract probably acted against acetaminophen-induced microsomal lipid peroxidation triggered during toxic hepatitis.

Covalent protein modification, and free radical damage due to acetaminophen metabolism have been identified as pathways leading to hepatocyte damage; however, evidence has also been published that TNF-α may participate in the processes causing liver failure, because the administration of anti-TNF-α antibodies to acetaminophen-treated animals ameliorated the enzyme leakage during the early phase of the intoxication[28,29].

Overdoses of acetaminophen depleted glutathione stores, leading to accumulation of NAPQI, mitochondrial dysfunction[30] and the development of acute hepatic necrosis. The depletion of glutathione enhances the expression of TNF-α[31]. TNFQ primes phagocytic NADPH oxidase to the enhanced production of oxygen-free radicals and contributes to liver damage[32]. Acetaminophen caused a moderate sinusoidal perfusion failure (-15%) and infiltration of neutrophils along with activation of nuclear factor-kappaB (NF-κB) and intercellular adhesion molecule-1 and cytokine-induced neutrophil chemoattractant-1 mRNAs.

Reactive oxygen species (ROS) activate NF-κB by releasing the inhibitory subunit from NF-κB complex[33]. Increase in the production of ROS during acetaminophen metabolism leads to activation of NF-κB and increase in the synthesis of TNF-α. The animals pretreated with *S. polycystum* extract showed considerable inhibition in the elevation of TNF-α, which may be attributed to the inhibition of excessive reactive metabolite by their

antioxidant nature.

The electron microscopic observation demonstrated the contribution of multiple factors toward the development of toxic hepatitis, and showed the damage in nuclear membrane with swelling of mitochondria in acetaminophen-intoxicated rats. The increase in lipid peroxides observed in microsomal membrane might be viewed as an additional index of alteration of membrane permeability, suggesting that oxidative modifications of unsaturated lipids are also detectable *in vivo* after AAP administration. The respiratory enzyme NADH dehydrogenase is located in the mitochondrial membrane[34]. NAPQI, the toxic metabolite of acetaminophen, arylates and oxidizes essential protein sulfhydryls in the mitochondrial respiratory chain, thereby limiting the ability of the mitochondria to meet the energy demand of the cell and affecting cellular energy homeostasis[35], which is consistent with our electron microscopic observation which depicts remarkable morphological alterations of mitochondria. The appearance of lipid droplets and sinusoids, concurrent with the evolution of hepatic necrosis in acetaminophen-induced rats, represents the adaptive response to the disturbance of phospholipid metabolism induced by potentially toxic stimuli. In addition, the acetaminophen-induced secondary lipidosis as reflected by the accentuated lamellated dense bodies is likely to result from the binding of the toxic metabolite with membrane phospholipids. The exact mechanism of the aforementioned relationship is still elusive. The rats pretreated with *S. polycsytum* considerably prevented the alterations in liver cell structural integrity triggered by acetaminophen, thereby showing that the extract contain bioactive compounds which may have a role in modulating the mitochondrial functional status and lipid metabolizing enzymes[36,37]

In conclusion, the defensive action of the seaweed extract may be due to their anti-hepatotoxic and antioxidant property against the acetaminophen-induced severe impairment in the drug metabolizing microsomal enzyme system and inhibitory action on TNF-α. The extract also possesses promising protection against acetaminophen-induced alterations in liver cell structural integrity during toxic hepatitis. Even though the extract possesses a promising preventive mode of efficacy against acetaminophen toxicity, the mechanism of its action is still elusive. Presently, we have isolated 8 fractions from the *S. polycystum* extract and their structural identification using nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC), and the mechanism of action in liver protection is under way.

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BASIC RESEARCH

Effect of liver regeneration after partial hepatectomy and ischemia-reperfusion on expression of growth factor receptors

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Abstract

AIM: To investigate the effects of experimental partial hepatectomy and normothermic ischemia-reperfusion damage on the time course of the expression of four different growth factor receptors in liver regeneration. This is relevant due to the potential therapeutic use of growth factors in stimulating liver regeneration.

METHODS: For partial hepatectomy (PH) 80% of the liver mass was resected in Sprague Dawley rats. Ischemia and reperfusion (I/R) were induced by occlusion of the portal vein and the hepatic artery for 15 min. The epidermal growth factor receptor, hepatic growth factor receptor, fibroblast growth factor receptor and tumour necrosis factor receptor-1 were analysed by immunohistochemistry up to 72 h after injury. Quantitative RT-PCR was performed at the time point of minimal receptor expression (24 h).

RESULTS: In immunohistochemistry, EGFR, HGFR, FGFR and TNFR1 showed biphasic kinetics after partial hepatectomy with a peak up to 12 h, a nadir after 24 h and another weak increase up to 72 h. During liver regeneration, after ischemia and reperfusion, the receptor expression was lower; the nadir at 24 h after reperfusion was the same. To evaluate whether this nadir was caused by a lack of mRNA transcription, or due to a posttranslational regulation, RT-PCR was performed at 24 h and compared to resting liver. In every probe there was specific mRNA for the receptors. EGFR, FGFR and TNFR1 mRNA expression was equal or lower than in resting liver, HGFR expression after I/R was stronger than in the control.

CONCLUSION: At least partially due to a post-transcriptional process, there is a nadir in the expression of the analysed receptors 24 h after liver injury. Therefore, a therapeutic use of growth factors to stimulate liver regeneration 24 h after the damage might be not successful.

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Key words: Partial hepatectomy; Liver ischemia; Growth factor receptors

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INTRODUCTION

Cytokines, hormones and growth factors are known to be involved in the liver's regeneration. Until now, therapeutic use of growth factors, or anything else capable of stimulating regeneration *in vivo,* has not been successful[1]. Since not only ligands like cytokines, hormones and growth factors but also receptors could limit the influence of these factors, the aim of our research was the kinetics of growth factor receptors in liver regeneration. Epidermal growth factor receptors (EGFR) and hepatocyte growth factor receptors /c-Met (HGFR) are activated immediately after partial hepatectomy (PH)[2,3]. Research with c-met knockout mice demonstrates impaired liver regeneration after PH or toxic injury[4,5]. However, it seems that other signal transduction pathways could compensate for the lack of HGFR and induce liver growth. Furthermore, impaired liver regeneration in complicated situations seems to be more the consequence of alterations of the receptor site of the signalling pathway rather than a result of a decreased amount of ligands. In a model of fulminant hepatic failure due to a combination of PH and right liver lobe necrosis, Mizuguchi *et al*[6] found lower c-met expression compared to PH alone, but elevated levels of cytokines. The same is true after hepatectomy in rats with CCL(4)-induced cirrhosis[7,8]. HGF over-expression when c-Met is not altered leads to tumour formation in transgenic mice but could not promote liver regeneration[8]. From these references we hypothesised, that growth factor receptors, more than their ligands, are limiting in liver

regeneration and therefore, it is more reasonable to look for the expression of the receptors.

There are several growth factor receptors, other than HGFR/c-Met, like EGFR, fibroblast growth factor receptor (FGFR) and tumour necrosis factor alpha receptor 1 (TNFR1) involved in liver regeneration about which, even less is known.As we know from recent experiments with transgenic animals, these receptors are required in liver regeneration[9,10]. Their kinetics during liver regeneration were unknown until now. Therefore, we analysed the kinetics of EGFR, HGFR, FGFR and TNFR1 in two models of liver regeneration: PH and reperfusion after warm 15 min ischemia (I/R).

MATERIALS AND METHODS

Models

The experiments were performed in male Sprague-Dawley rats weighing 350-500 g (Charles River, Sulzfeld, Germany) in accordance with the local regulation for animal experiments. All procedures were performed under isoflurane (Abbott, Wiesbaden, Germany) inhalation anaesthesia 2.5%-4%.

Partial hepatectomy (PH) was conducted after midline laparotomy. About 80% of the liver mass was resected, following ligation with a 3-0 polyglycolic acid ligature (Dexon,Braun, Melsungen, Germany). Then, the incision was closed by using a continuous two-layer suture technique.

Ischemia and reperfusion (I/R) were induced after midline laparotomy, by occlusion of the portal vein and the hepatic artery near the liver, by a vessel loop (Ethicon, Norderstedt, Germany). Care was taken not to occlude the bile duct. After 15 min, the loop was removed and the incision closed.

In each of the two models, regeneration after the injury was analysed at the following time points 0 h, 5 h, 2 h, 6 h, 12 h, 24 h, and 72 h. Each group consisted of five animals.

After regeneration, euthanasia was performed under general anaesthesia by blood collection and hepatectomy.

Immunohistochemistry

Receptors were identified by immunohistochemistry using 4 μm paraffin sections cleared with Rotihistol (Roth, Karlsruhe, Germany), passed through a graduated series of alcohol, and incubated for 5 min in room temperature water to rehydrate. Antigen retrieval for EGFR was accomplished using a microwave at 750 watts in target retrieval solution (pH 9.9) (vectastain, vectorlab, Burlingame, USA) and for TNFR1, HGFR, FGFR by incubation in 0.05% proteinase K for 15 min. Endogenous peroxidase activity was halted using 3% peroxide in methanol for 30 min, followed by endogenous biotin bound to avidine for 10 min and finally, in biotin for 10 min; non-specific binding was blocked by 1% BSA (Sigma, Munich, Germany). The antibodies for staining receptors were anti-TNFR1 (H-5, Santa Cruz Biotechnology, Santa Cruz, USA), anti-EGFR (1161-1186,Calbiochem, San Diego, USA), anti-Met (B-2; Santa Cruz Biotechnology, Santa Cruz, USA), and anti-FGFR (Ab-1, Oncogene, Boston, USA). Antibodies were used at a dilution of 1:50-1:100 in PBS. Detection was performed using an avidine -biotin peroxidase system (vectastain, vectorlab, Burlingame, USA) with anti-mouse or anti-rabbit antibodies, and stained with 3-amino-9-rthyl-carbazole (Carbazol, Sigma, Munich, Germany).

Cell preparations for PCR

Zone-selective cell isolation was performed after periportal or perivenous cell damage by ante- or retrograde perfusion with digitonin as described by Lindros[11].The portal vein and vena cava superioris were canulated under anaesthesia and washed out with approx. 300 mL oxygenated buffer I (5.36 mmol/L KCl, 1.17 mmol/L $MgSO_4$, 0.79 mmol/L Na_2HPO_4, 0.15 mmol/L KH_2HPO_4, 10 mmol/L Hepes, 145 mmol/L NaCl, 0.1% glucose, and 1 mmol/L $CaCl_2$, at pH 7.4). Afterwards, the liver was perfused via the portal vein or vena cava with 4 mM digitonin in oxygenated buffer I for 1-1.5 min. In a third step, the liver was washed out with 900 mL buffer II (5.36 mmol/L KCl, 0.77 mmol/L $MgSO_4$, 0.93 mmol/L $MgCl_2$, 0.34 mmol/L Na_2HPO, 0.44 mmol/L KH_2HPO, 0.2 mmol/L EDTA, 10 mmol/L Hepes, and 145 mmol/L NaCl, at pH 7.4). Afterwards, the liver was perfused with collagenase (0.025%) in buffer III (5.36 mmol/L KCl, 0.77 mmol/L $MgSO_4$, 0.93 mmol/L $MgCl_2$, 0.34 mmol/L Na_2HPO_4, 0.44 mmol/L KH_2HPO_4, 10 mmol/L Hepes, 145 mmol/L NaCl, 0.2% glucose, 0.003% penicillin, 0.005% streptomycin, and 0.2% BSA, at pH 7.4), and one minute later, 4.3 mL of 200 mmol/L $CaCl_2$ was added.

After mincing the liver into small particles in oxygenated buffer I, the liver cell-buffer mixture was pressed through a gauze and then through a 100 μm nylon filter. The cells were washed twice with 50 mL oxygenated buffer and centrifuged at 500 rpm for 5 min at 4℃. The pellet was brought to a Percoll gradient (Percoll, Amersham Biosciences d = 1.065) and centrifuged at 500 rpm for 15 min at 20℃ . Hepatocytes were collected in the pellet and washed twice with 50 mL oxygenated buffer.

Gluminsynthetase (GS) assay

The correct discrimination between PP and PC hepatocytes was checked by performing the GS assay, as GS is located strictly pericentrally[12,13] . 250 000 hepatocytes were homogenized in oxygenated buffer I. A 100 μL sample was added to 400 μL test mix (2 × 250 mmol/L imidazole buffer, 2 × 250 mmol/L l-glutamic acid, 1 × 250 mmol/L hydroxylamine hydrochloride, 1 × 1.6 mmol/L adenosine-5-diphosphate, 1 × 250 mmol/L disodiumhydrogen arsenate, 1 × 10 mmol/L manganese chloride) and incubated 16-60 min at 37℃. The reaction was stopped by 100 μL stop-mix (2.42% iron III chloride, 1.45% trichloric acid in 5.4% HCl). The mixture was centrifuged (15 min, 3300 g) and analyzed in a photometer at 540 nm. GS activity was calculated as follows:

GS activity $= \Delta E \times V \times 1000 \times (\varepsilon \times d \times \Delta t \times v \times c)^{-1}$ U/g with E = extinction, V = 1.5 mL

$\varepsilon = 0.04395\ l^* mmol^{-1^*} mm^{-1}$, d = 10 mm , t = time, v = 0.1 mL, c = protein concentration.

PC hepatocytes were used with a GS activity above 300 U/g, PP hepatocytes were used with a GS activity below 30 U/g.

RT-PCR

RNA was isolated using the RNeasy kit (QIAGEN, Hilden, D) and quantitative PCR was performed using

Table 1 Expression of growth factor receptors in immunohistochemistry after partial hepatectomy

	Resting liver	PH						I/R					
Time (h)	0	0.5	2	6	12	24	72	0.5	2	6	12	24	72
EGFR	-	+	+[1]	++	+	-	+	-	-	-	+	-	-
FGFR	(+)	(+)	+[2]	++	++	(+)	+	(+)	(+)	-	-	-	-
HGFR	-	-	+	++	++	-	++	-	+	+	+	-	-
TNFR1	(+)	(+)	+	+	(+)	(+)	++	(+)	(+)	(+)	(+)	-	+

(PH), 15-min warm ischemia (I/R). -: negative, (+): positive but not more than in the resting liver, +: positive, ++: strong positive; [1]: mainly portal; [2]: mainly centre of the hepatic acinus.

the SYBER-Green technique (QuantiTect, QIAGEN, Hilden, D), according to the instructions given by the manufacturer. PCR was carried out by a one-step RT-PCR with the following cycle conditions: cycle 1: Temp.: 50℃ 30:00 min, cycle 2: Temp.: 95℃ for 15:00 min, cycle 3: Temp.: 94℃ for 0:15 min, cycle 4: Temp.: 52℃ for 0:30 min, cycle 5: Temp.: 72℃ for 0:30 min, cycles 3-5 were repeated 45 times. Primer design was performed by Primer 3 (primer3_www.results.cgi) with sequences from NCBI:
HGFR (NM-031517): gtcttcaagtagccaagggc and aacatgcagtttcttgcagc
FGFR (NM-024146): gaagagcgacttccatagcc and acactgttacctgtctgcgg
TNFR1 (BC 086413): ctttgagcctttctaacccg and agtaagacgatcggaccagg
EGFR (M37394): tggacaaccctcatgtatgc and acatagtccaggaggcaacc

As an internal standard, we used 18 s rRNA: cgt agt tcc cga cat aaa cg and cag ctt tgc aac cat act cc. The difference between the threshold cycle C_T of 18 s rRNA and the mRNA of interest is shown as ΔC_T. A high ΔC_T represents a low amount of specific RNA and vice versa.

Statistics

Data management and *t*-test for unpaired data were carried out using PRISM® software. In case of semi-quantitative analysis in immunohistochemistry, a statistical analysis was omitted.

Unless otherwise stated, the chemicals used were from Sigma, Munich, Germany.

RESULTS

Using immunohistochemistry, we analysed the expression of EGFR, FGFR, HGFR and TNFR1 in the resting liver (tissue not influenced by proliferative stimuli) as control, during liver regeneration after partial hepatectomy, and during reperfusion after 15-min normothermic ischemia. Receptor expression was analysed semi-quantitatively with results shown in Table 1. For exemplary immunohistochemistry see Figures 1 and 2. There is weak expression of FGFR but a strong one by TNFR1 in resting liver. Following PH, there is a strong expression of all receptors after 6 h and 12 h, and again after 72 h. Yet, there is no, or in case of FGFR and TNFR1, only weak expression of the receptors after 24 h. During regeneration after I/R, there is only a short and weak expression of EGFR, HGFR and TNFR1 in the first 12 h. TNFR is expressed strongly after 72 h again. After PH, as after I/R, there was no expression of any receptor, stronger than in the control after 24 h.

As there was a gap with no expression of any receptor, at 24 h after the liver injury, even when there was positive immunohistology at 72 h, we performed a quantitative PCR comparing the receptor mRNA in the resting liver and at 24 h. Each PCR was separately conducted for periportal and pericentral hepatocytes. As

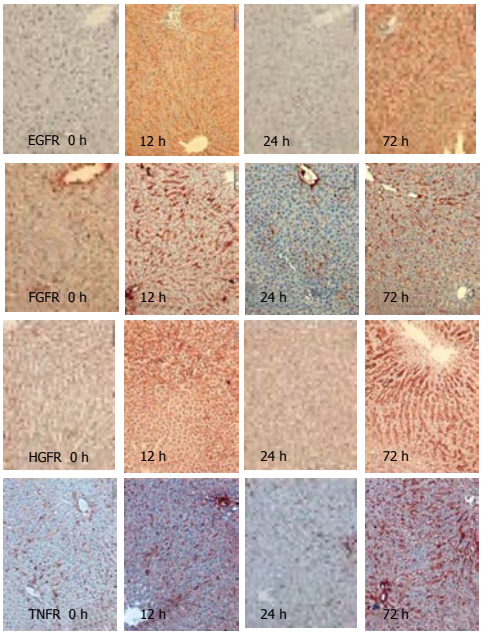


Figure 1 Sample immunohistochemistry of EGFR, FGFR, HGFR and TNFR in regenerating liver 12 h, 24 h, and 72 h after partial hepatectomy. (0 h: control).

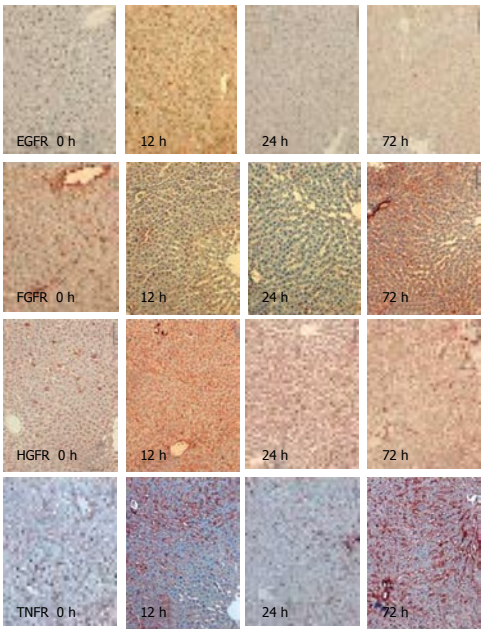


Figure 2 Sample immunohistochemistry of EGFR, FGFR, HGFR and TNFR in regenerating liver 12 h, 24 h, and 72 h after ischemia and reperfusion. (0 h: control).

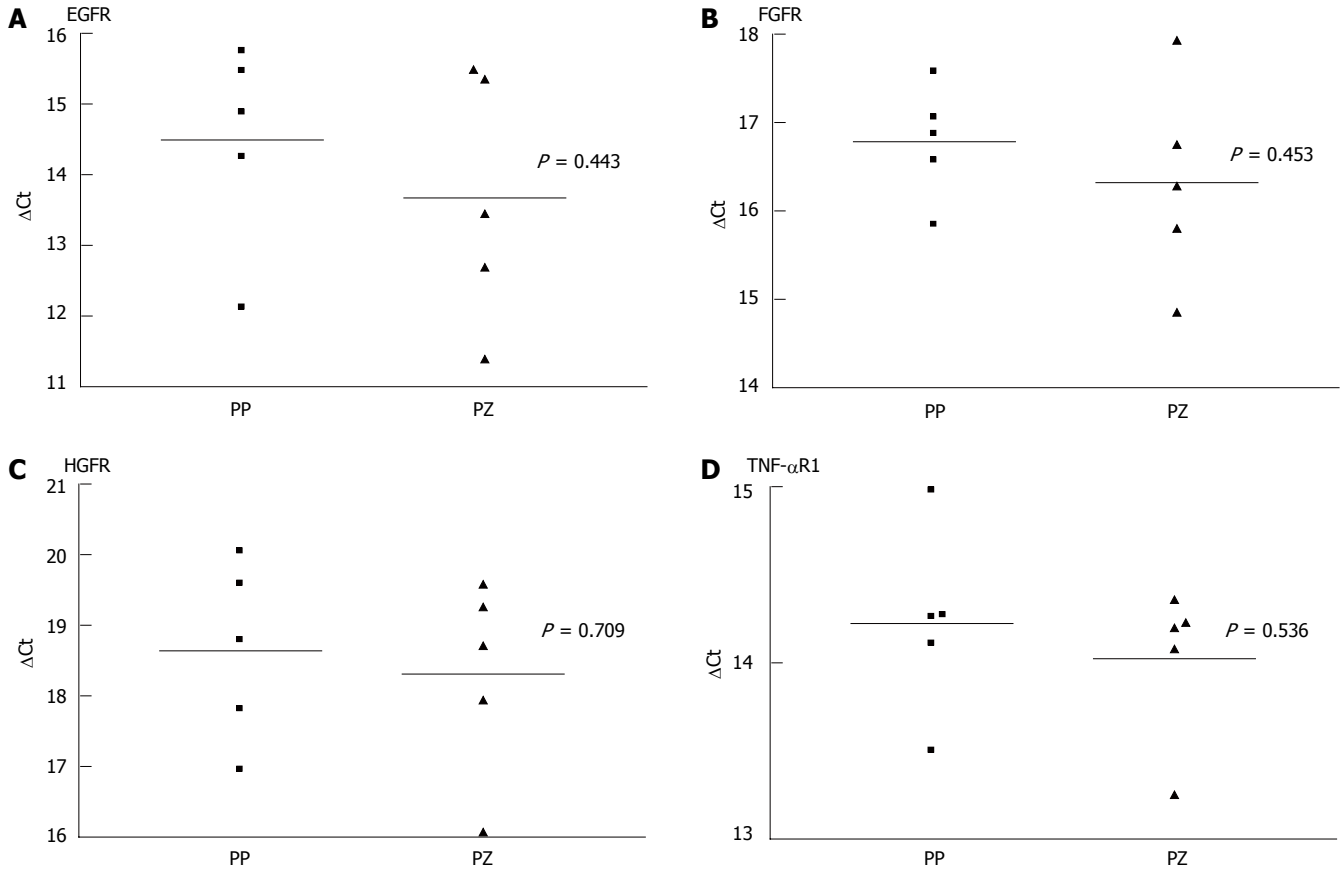


Figure 3 Results of quantitative PCR comparing receptor expression in periportal (PP) and pericentral (PC) hepatocytes in resting liver.

shown in Figures 3 and 4, there was no difference in the expression of the analysed mRNA between periportal and pericentral hepatocytes in the resting liver or in regenerating liver after PH. In I/R there was a stronger pericentral expression of receptors. In case of EGFR and TNFR1 mRNA, this difference was significant (Figure 5).

Comparing receptor mRNA between resting liver and in regenerating liver, there was a significantly reduced expression of EGFR after PH and I/R, FGFR after I/R and TNFR1 after PH, represented by a greater ΔC_T. Only HGFR was expressed more strongly 24 h after I/R than in resting liver. At every time point, there was a positive expression of receptor mRNA, even if immunohistochemically no receptor could be detected .

DISCUSSION

We analysed the expression of several growth factor receptors in two different models of liver injury and found a marked expression after 6 and 12 h with a nadir at 24 h and an increased expression after 72 h.

With no receptors to interact with, external growth factors might therefore not be able to promote the course of liver regeneration *in vivo*[1] when administered at the time of the nadir, at 24 h. It furthermore should be discussed, whether external growth factors influence receptor expression by negative feedback mechanisms. At 72 h, when the hepatocyte proliferation reaches its maximum, there is, once again, a (weak) expression of receptors. During this period, external growth factors do not promote regeneration, probably because proliferation is already at its maximum. Further research, investigating the meaningful application of growth factors in liver regeneration, is clearly warranted and should consider these two hypotheses.

The results demonstrated here correlate to previously published data of EGF binding studies. There, the observation was made that the EGF binding capacity of hepatocytes was reduced after PH, compared to control[14]. It is not yet clear why receptor kinetics during liver regeneration, after PH, are a biphasic process. Based on immohistochemical findings at 6 h after injury, we assume that there is an immediate expression of preformed receptors. It appears unlikely that *de novo* receptor synthesis can take place within 6 h. Later, after the injury, a *de novo* rebuilding of receptors might occur which correlates to the increase after 72 h.

After I/R, the expression of receptors is only very weak, only slightly more intensive than in the resting liver. Interestingly, the biphasic kinetics of TNFR1 after I/R are comparable to PH. In fact, the expression of FGFR and TNFR1 could be suppressed beyond the baseline using I/R.

The reason for the lack of receptor expression at 24 h after injury could be reduced mRNA expression, a posttranscriptional mechanism or shedding of the receptors. All of these mechanisms have been described in the context of receptor regulation processes[15,16]. In all samples, there is mRNA expression, yet at a reduced level. Therefore, a negative posttranscriptional effect may

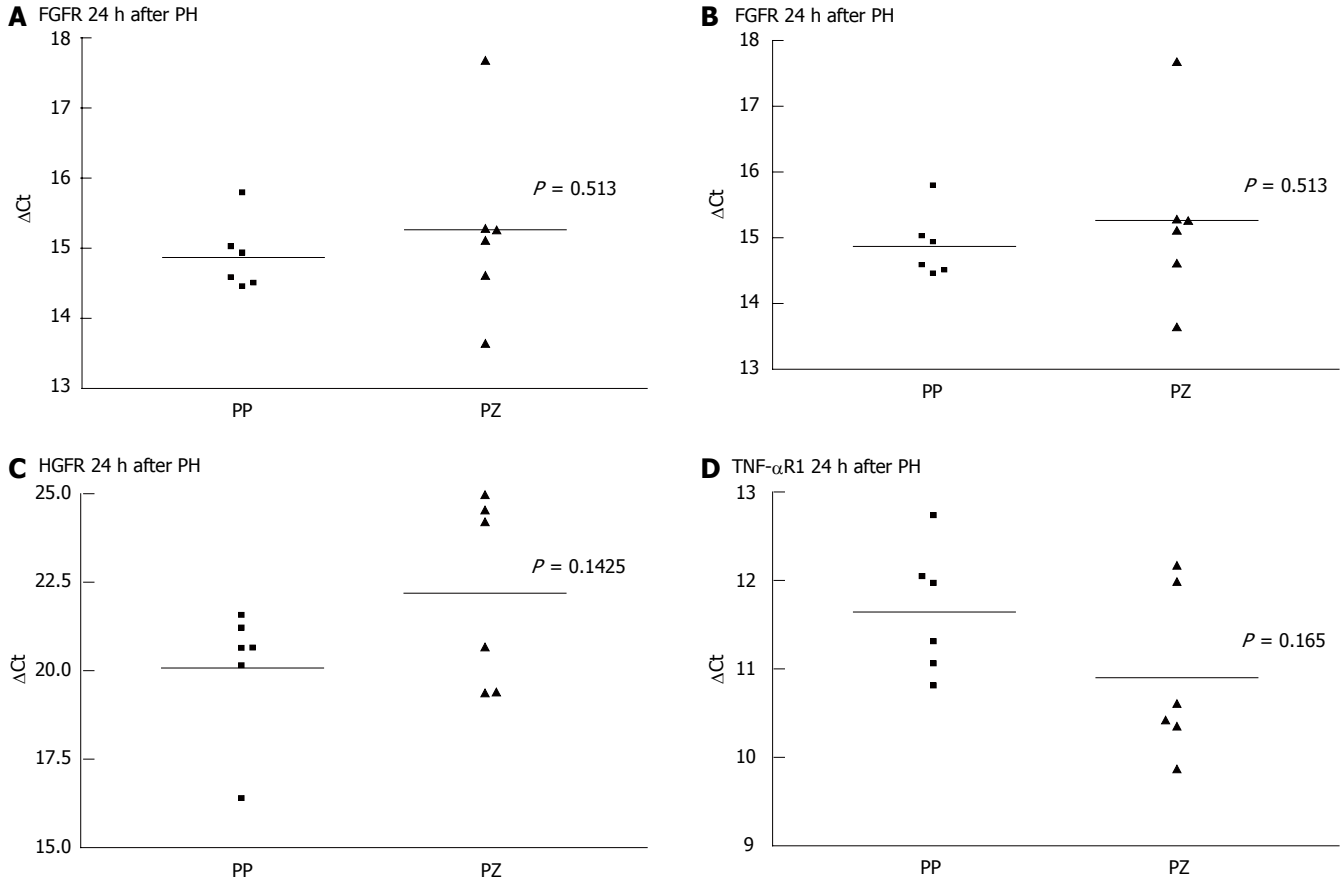


Figure 4 Results of quantitative PCR comparing receptor expression in periportal (PP)and pericentral (PC) hepatocytes 24 h after liver injury.

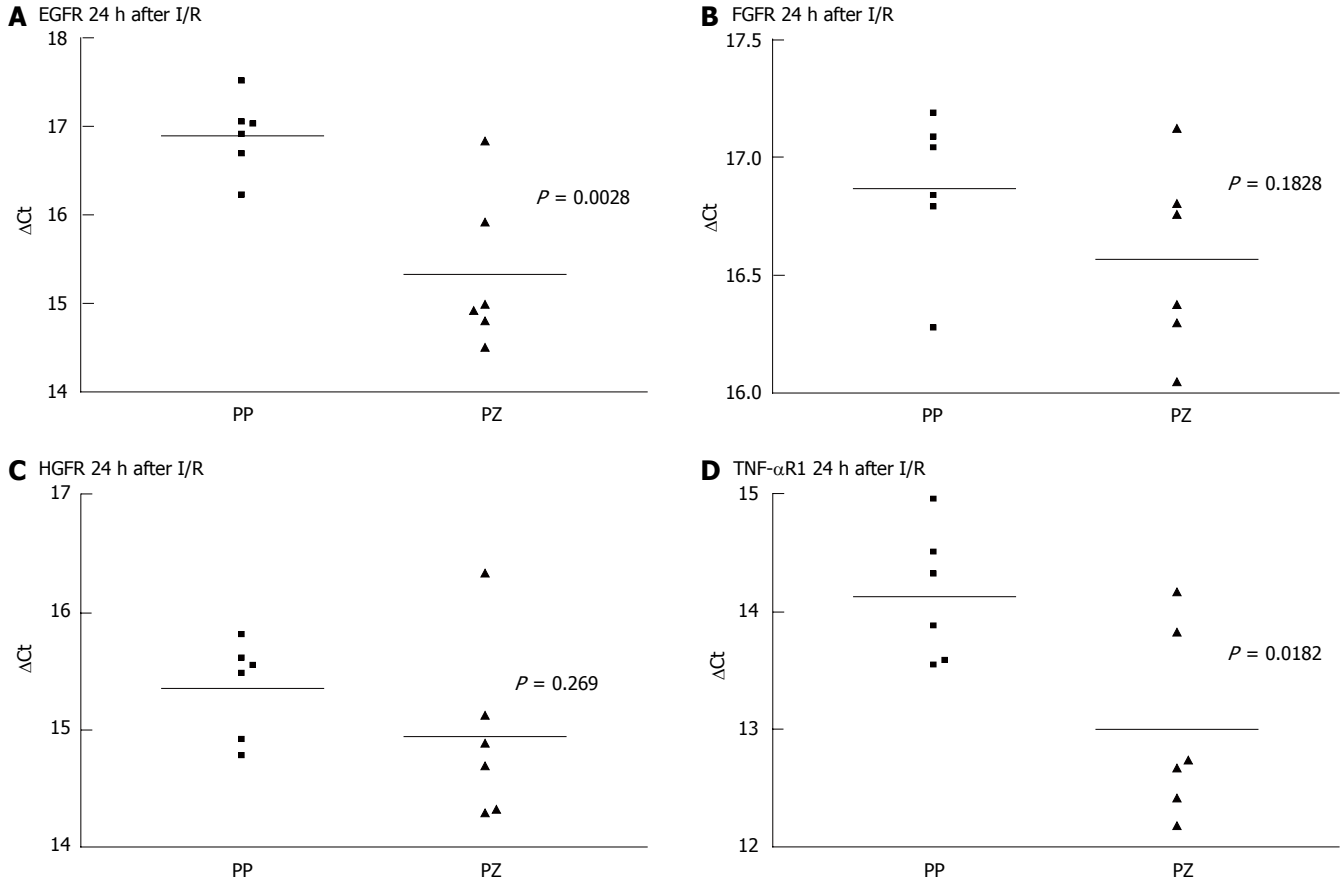


Figure 5 Results of quantitative PCR comparing receptor expression in periportal and pericentral hepatocytes 24 h after reperfusion.

take place as well as a reduced translation of mRNA. In case of HGFR after I/R, this posttranscriptional effect is demonstrated, as more specific mRNA is detectable in regenerating liver tissue than in control.

In I/R model, we did not find the same increase in receptor expression as after PH. This is of particular interest because in myocardial and neuronal tissue, an up-regulation of growth factor receptors seems to be involved in ischemic preconditioning[17,18]. We could not find a similarly strong receptor expression after ischemic stimuli in liver regeneration. Although ischemic preconditioning is effective in the clinical setting of partial hepatectomy, it might not be due to a strong expression of growth factor receptors. As opposed to the resting liver and to the regenerating liver after PH, there is a zone-specific difference in the receptor mRNA expression after I/R. There is stronger expression in pericentral than in periportal hepatocytes. In immunohistochemistry, this difference is not found since there are no receptors at all. The observed variations in mRNA expression reflect the different impact of ischemia on the various parts of the liver acinus. The pericentral part is more sensitive than the periportal. In regeneration after I/R, there is stronger receptor mRNA expression in pericentral hepatocytes. Contrary to mRNA expression in I/R, there is no obvious predilection to any part of the liver acinus in the PH model, neither concerning the trauma nor the receptor expression (immunohistochemically and mRNA) during regeneration. This is of interest, as we know[19] that the proliferation after PH is stronger in periportal than in pericentral areas.

At 24 h after partial hepatectomy, many regulatory processes take place in the regenerating liver. As Xu *et al*[20] demonstrated by microarray, there are more genes expressed at 24 h than at any other time during liver regeneration. The receptors analysed here are not up-regulated at this time point, maybe they are part of the 135 genes Xu *et al* found to be down-regulated. As there should be a posttranscriptional regulation in case of the receptors, the approach of Liu *et al*[21], for analysing proteomics of regenerating liver, should be even more interesting than microarrays.

CONCLUSION

Concerning the expression of growth factor receptors, liver regeneration is a biphasic process with a nadir at 24 h. As a result, we hypothesise that the expression of receptors is an essential regulative factor in this process. This will have to be validated in subsequent studies.

Further research must focus on the mechanisms responsible for the decrease in receptor expression after 24 h. It should also be promising to analyse the expression of growth factor receptors in other models like sepsis or combined injuries such as, resection plus reperfusion or resection plus sepsis, since these are combinations correlated to complicated courses of liver regeneration in the clinical setting. In order to use therapeutic growth factors in liver regeneration, detailed knowledge about the kinetics of their corresponding receptors is of elementary importance.

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BASIC RESEARCH

Isolation, characterization and culture of Thy1-positive cells from fetal rat livers

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Abstract

AIM: To investigate whether Thy1 recognizes oval cells in the fetal liver and to characterize the cultured Thy1-selected cells from E14 rat livers.

METHODS: Thy1 populations were analyzed by fluorescence activated cell sorter analysis. Thy1 positive cells were isolated using magnetic beads. Hepatic markers were detected by Western blotting, immunocytochemistry and RT-PCR.

RESULTS: The percentage of Thy1-positive cells decreased during early development of fetal rat liver (E13-E16). E14 fetal livers contained 7.8% Thy1 positive cells, of which 61% were positive for α-fetoprotein (AFP) and 25% expressed albumin. The Thy1+ population expressed oval cell markers c-Kit and CXCR4, liver enriched-transcription factors HNF1α and HNF6, hepatocytic markers albumin, AFP and cytokeratin 18, and biliary marker cytokeratin 19. Thy1- selected cells formed only mesenchymal colonies when plated on collagen and in serum-containing media. Thy1 selected cells were able to form hepatic colonies positive for HNF1α, HNF6, albumin, AFP, cytokeratin 18, cytokeratin 19 and glycogen, when grown on STO feeder layers in serum free-media.

CONCLUSION: Oval cells positive for Thy1 are present in early liver embryonic stages.

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Key words: Fetal liver; Oval cells; Thy1

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INTRODUCTION

Bipotential hepatic stem cells are found in murine and rat embryonic livers at stages E13 and E14, although at these embryonic stages a large majority of cells are already committed to the hepatocytic and bile duct lineage. Bipotential liver cells or hepatoblasts express an array of genes, such as α-fetoprotein (AFP), albumin, gamma-glutamyl transpeptidase (GGT), glutathione-S-transferase p and HNF6[1-3]. With maturation towards the hepatocytic lineage, fetal liver parenchymal cells have lower levels of AFP and higher levels of albumin, start expressing cytokeratin 18 (CK18) and break loose of GGT expression. Cells that differentiate towards biliary epithelial lineage retain GGT expression, express cytokeratins 7 and 19, c-kit and CD34 and stop expressing AFP and albumin[1-5]. Some liver-specific genes, such as glutamine synthetase, tyrosine aminotransferase (TAT), phosphoenolpyruvate carboxykinase and α2-microglobulin appear later in hepatocytes, at birth or even after birth. Recently, several groups have isolated bipotential fetal progenitor cells from murine and rat origin, which form colonies of both hepatocytes and bile duct cells, identified by albumin and cytokeratin 19 (CK19) expression, respectively[6,7].

There is controversy over the identity of adult liver stem cells, and whether they exist in the adult liver. One of the candidates is the oval cells. However, oval cells are only detected after certain protocols, such as carcinogenic insults to the liver, followed by partial hepatectomy[8]. There are suggestions that oval cells are bone marrow cells that migrate to the liver and become hepatocytes. Reconstitution of sublethally-irradiated male mice with female bone marrow, followed by a protocol that results in oval cell proliferation, showed that oval cell-derived hepatocytes are of donor origin[9]. Moreover, the homing molecule for hematopoietic stem cells to the bone marrow, stroma-derived factor 1 (SDF-1), is induced on hepatocyte surface in livers treated with oval cell proliferation-inducing protocols, and the oval cells express the CXCR4, the receptor for SDF-1[10]. If indeed oval cells are of bone marrow origin, it may explain the expression of common markers in both oval cells and hematopoietic cells, such as

Thy1, CD34 and c-kit. Several current studies have shown that bone marrow cells that arrive at the liver do not differentiate into hepatocytes, but undergo fusion with hepatocytes[11].

Recently, one group has investigated the presence of oval cells in the fetal liver, since expression of Thy1 is never determined in fetal liver hepatoblasts[12,13]. This group isolated Thy1 positive cells from E16-E20 livers, and stained these cells with hepatocytic marker CK18[12]. They found that 0.5%-1% of the cells are positive for both Thy1 and CK18 and speculated that these double-positive cells may be hepatic progenitor cells[12]. Other markers for oval cells are c-kit, oncostatin M receptor β and CXCR4 and dlk[10,14,15]. Dlk is a marker for both murine hepatoblsts and murine oval cells, and its expression is the strongest at E10.5 and down regulated after E16.5[16].

The purpose of the present study was to determine whether oval cells are present in early embryonic fetal liver, and to characterize the cultured Thy1- positive cells from E14 fetal livers.

MATERIALS AND METHODS

Animals

Fischer 344 rats with known durations of pregnancy were maintained in the animal facility of the Tel Aviv Sourasky Medical Center on a standard rat chow diet in a 12 h light and dark cycle. By convention, the first day of gestation was defined as d 0. Use of animals was in accordance with the NIH policy on the care and use of laboratory animals and approved by the Animal Care and Use Committee of the Tel Aviv Sourasky Medical Center.

Isolation of fetal rat hepatocytes

Cell suspensions enriched for liver parenchymal cells were isolated as described previously with minor modifications[17]. Livers from E13, E14 and E16 embryos were removed and placed into ice cold, Ca^{2+}-free Hank' s balanced salt solution (HBSS) containing 0.8 mmol/L $MgCl_2$ and 20 mmol/L HEPES (Sigma Chemical Company, Israel), pH 7.3 (modHBSS). After all tissues were collected and non-hepatic tissue was removed, HBSS-5 mol/L EGTA was added to a final EGTA concentration of 1 mmol/L. The livers were gently triturated 6-8 times to partially disaggregate the tissue and then centrifuged at 400 r/min for 5 min at 4℃ (all subsequent centrifugation steps were done at the same settings). The pellet of cells and tissue were resuspended in 35 mL modHBSS containing 0.08 g collagenase type 1 (Worthington Biochemical Corporation, NJ, USA) and 1 mmol/L $MgCl_2$, gently triturated and stirred at 37℃ for 15 min in an Erlenmeyer flask. The dispersed cells were pooled, centrifuged and resuspended in modHBSS. Cell number and viability were determined by hemacytometer and trypan blue exclusion.

Fluorescence activated cell sorter analysis

The freshly isolated liver cells from fetal livers were stained for 1 h on ice with Thy1-fluorescein isothiocyonate (FITC) (Serotec, Oxford, United Kingdom) diluted in DMEM + 100 mL/L fetal calf serum (FCS) (Biological Industries, Bet Haemek, Israel). At the end of the incubation, the cells were washed and fixed in 5 g/L paraformaldehyde in phosphate buffered saline (PBS). Thy1-positive cells were analyzed using a Becton-Dickson fluorescence activated cell sorter (FACS) and the results were analyzed using the WinMDI software. For double staining of Thy1 and α-fetoprotein, the cells were stained with Thy1-phycoerythrin (PE)(1:100), fixed with solution A of Intrastain kit (Dako, Glostrup, Denmark), permeabilized with solution B, and stained for intracellular expression with antibodies to rat α-fetoprotein-FITC (1:80) (Nordic, Tilburg, The Netherlands). Thy1 and α-fetoprotein were analyzed using a Becton-Dickson FACS and the results were analyzed using CellQuest software. For double labeling of cell surface with dlk and oncostatin receptor β, and intracellular staining of AFP, we used antibodies to dlk (Santa Cruz Biotechnologies, Santa Cruz, CA) and to mouse OSMRβ (R&D Systems, Minneapolis, MN), followed by anti-goat-PE conjugated antibody (Jackson Laboratories, Bar Harbor, ME) and staining with AFP-FITC.

Thy1 selection

The freshly isolated fetal liver cells were selected using an EasySep kit from StemCell Technologies, Vancouver, Canada. Briefly, cells were incubated for 15 min at room temperature with 3 mg/L Thy1-FITC (Serotec, Oxford, United Kingdom) in PBS containing 20 mL/L FCS and 1 mmol/L EDTA. Cells were washed once with excess buffer, then incubated with EasySep FITC selection cocktail for another 15 min, and then with the EasySep magnetic nanoparticles for 10 min. Buffer was added to cells at total volume of 2.5 mL, and the labeled cells were placed for 5 min inside the Violet Magnet (StemCell Technologies, UK). Unlabeled cells were poured off from the tube. The procedure was repeated two more times. The selected cells were resuspended in buffer and counted, and used for further characterization or for plating.

Clonal assays

Thy1-selected cells were plated at two densities per well in 6- well plates (approximately 1000 and 2000 cells/cm^2). The plates were coated with collagen type I. The media used to culture the cells were as follows. Medium 1: DMEM supplemented with 100 mL/L FCS (Biological Industries, Bet Haemek, Israel), 50 μg/L EGF (PeproTech Asia/CytoLab, Rehovot, Israel) and 10 mg/L insulin (Sigma, St. Louis, MI)[18]; medium 2: DMEM supplemented with 10 μg/L oncostatin M and 10 μg/L flt-3 ligand (PeproTech Asia/CytoLab, Rehovot, Israel)[19]; medium 3: DMEM supplemented with 100 mL/L FCS, 1mg/L insulin, 10^{-7} mol/L dexamethasone, 10 mmol/L nicotinamide, 50 μmole/L β-mercaptoethanol, 50 μg/L HGF (PeproTech Asia/CytoLab, Rehovot, Israel), 20 μg/L EGF[6]; medium 4: DMEM supplemented with 100 mL/L FCS.

The cells were allowed to grow for 2 wk with the medium changed every three days. The colonies were stained with 2.5 g/L crystal violet solution and counted. The experiments were done at least three times.

STO feeder layers

STO cells were a kind gift of Professor Lola Reid (UNC, Chapel Hill, NC). The cells were cultured in DMEM (F12 media 1: 1) supplemented with 100 mL/L FCS, 10 mL/L dimethylsulfoxide (DMSO), 100 mg/L penicillin and 100 mg/L streptomycin.

The STO cells were growth arrested for 3 h with 10 mg/L mitomycin C (Sigma, St. Louis, MI), then plated on 1 g/L gelatin-coated plates at a density of 60×10^3 cells/cm^2 in the medium described above and cultured for 2 d prior to seeding Thy1- selected cells on them.

Culture of Thy1- selected cells on STO feeders

Thy1-selected cells from e14 fetal livers were plated on STO feeder layers at a density of 4000 cells/cm^2 and cultured for 2 wk in hormonally defined medium (HDM)[20] consisting of DMEM supplemented with 100 mg/L penicillin/streptomycin, 2 g/L bovine serum albumin, 610 mg/L nicotinamide, 740 μg/L $ZnSO_4 \times 7H_2O$, 20 μg/L $CuSO_4 \times 5H_2O$, 5 mmol/L glutamine, 5 mg/L insulin, 5 mg/L iron-saturated transferrin, 5 μg/L selenous acid and 10^{-7} mole/L dexamethasone. All the medium additives were obtained from Sigma, St. Louis, MI.

Western blots

Total protein was extracted by incubation for 30 min on ice in lysis buffer (250 mmol/L sucrose, 5 mmol/L $MgCl_2$, 10 mmol/L Tris pH 8.0, 5 g/L Triton X-100 and 1 mmol/L PMSF), and insoluble material pelleted at 17 000 g. The extracts were normalized to total protein content, determined using Bradford reagent (Sigma, St. Louis, MI) and boiled for 5 min in sample buffer containing SDS and β-mercaptoethanol. Protein (10-20 μg per lane) was separated by SDS-PAGE (BioRad Protean II minigel) in 100 g/L acrylamide gels and blotted onto Hybond C extra (Amersham, Arlington Heights, IL) for 1 h at 250 mA constant current. The blots were blocked overnight in Tris-saline buffer pH 7.5, containing 0.5 g/L Tween-20 and 50 g/L milk, and incubated with antibodies to rat albumin (Sigma, St. Louis, MI) and AFP (Santa Cruz Laboratories, Santa Cruz, CA), washed and incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, ME). The blots were then subjected to chemiluminescent detection and flurorography using X-ray film (NEN Life Science Products, Boston, MA).

Immunocytochemistry

Cells were fixed with 95 mL/L ethanol for 10 min at 4℃, and incubated with a blocking solution containing 100 mL/L FCS for 30 min at room temperature. Cells were then incubated with primary antibodies to rat albumin (1:400) (Sigma, St. Louis, MI), mouse AFP (1:200), CK19 (Novacastra) for 1 h at room temperature, washed three times, followed by addition of anti-rabbit second antibody conjugated to Cy3 (1:100) (Jackson Laboratories, Bar Harbor, ME) for another hour at room temperature. Cells were washed three times and stained cells were visualized under Axiovert 25 microscope (Zeiss, Germany) with fluorescent attachments.

Glycogen detection

Glycogen granules in the cultured cells were detected using a periodic acid-Schiff (PAS) staining kit according to the manufacturer's instructions (Sigma, St. Louis, MI).

RT-PCR for liver specific markers

Total RNA was extracted from freshly isolated E14 and Thy1+ selected cells using EZ RNA kit (Biological Industries, Bet Haemek, Israel), treated with DNAse and cDNA was reverse-transcribed. For reverse transcription, 1-2 μg of total RNA was used. The cDNA obtained was used for PCR, using the following oligonucleotide primers[21]: HNF-1α: the 5' primer is 5' -AGCTGCTCCTCCATCATCAGA -3', the 3' primer is 5'-TGTTCCAAGCATTAAGTTTTCTATTCTAA -3'; HNF4α: the 5' primer is 5'-CTTCCTTCTTCA-TGCCAG -3', the 3' primer is 5'-ACACGTCCCCAT-CTGAAG -3'; HNF6: the 5' primer is 5' -AACTCCCAGCAAG-GACTTCCCCACT-3' , the 3' primer is 5'-CCATCTGCCCTGAAT-TACTTCCATTGC-3'; cytokeratin 18 (CK18): the 5' primer is 5'-GCCCTGGACTCCAGCAACT -3' and the 3' primer is 5'-ACTTTGCCATCCACGACCTT -3'; cytokeratin 19 (CK19): the 5' primer is 5' -ACCATGCAGAACCTGAACGAT- 3' and the 3' primer is 5'- CACCTCCAGCTCGCCATTAG-3' ; albumin: the 5' primer is 5'-CTGGGAGTGTGCA-GATATCAGAGT- 3' and the 3' primer is 5' -GAGAAG-GTCACCAAGTGCTGTAGT-3' ; alpha-fetoprotein: the 5' primer is 5' -GTCCTTTCTTCCTCCTGGAGAT- 3' and the 3' primer is 5'-CTGTCACTGCTGATTTCTCTGG-3'; oncostatin M receptor: the 5' primer is 5'-CCCCTGTGAG-GCCGAGGACCGGC-3' and the 3' primer is 5' -TGGCTTTACAATTGATGTTTGTCCCTG-3' ; stroma-derived factor 1(SDF1): the 5' primer is 5' –ATTCTTTGAGAGCCATGTCGC-3' and the 3' primer is 5'-CCTTGAGCTGAGTGACTCTCG-3' ; C-kit: the 5' primer is 5'-AGCAAGAGTTAAC-GATTCCGGAG-3' and the 3' primer is 5' -CCAGAAAGGTGTAAGTGCCTCCT-3'; CXCR4: the 5' primer is 5'-CCCTCGAGATGGAA-ATATACAC-3' and the 3' primer is 5'-GCTCTAGATTAGC-TGGAGTGAAA-3'. For glyceraldehydehyde-3-phosphate-dehydrogenase (GAPDH), the 5' primer is 5' -ACCACAGTCCATGCCATCAC-3' and the 3‘ primer is 5'-TCCACCACCCTGTTGCTGTA-3'. For PCR, 1.5 μL of the reverse transcription reaction was used, and the reaction mixture also contained 0.4 μmol/L of each primer, 200 μmol/L dNTP, 10 mmol/L Tris-HCl (pH = 8.3), 50 mmol/L KCl, 1.1 mmol/L $MgCl_2$, 0.1 g/L gelatin and 2.5 U RED Taq DNA polymerase (Sigma, St. Louis, MI). The mixture was denatured for 1 min at 94℃. The PCR was started at 94℃ for 10 s for the denaturation step, at 53℃ for 30 s for the annealing step and at 72℃ for 1 min for the elongation step. Thirty-five cycles were used to amplify the hepatic markers. The PCR products were finally separated in a 20 g/L agarose gel containing ethidium bromide.

Table 1 FACS analysis of cell surface markers in various rat fetal liver developmental stages

Embryonal stage	Thy1 positive cells (%)	Dlk positive cells (%)	OSM receptor β positive cells (%)
E13	10.5 ± 2.0	10.8	5.3
E14	7.8 ± 0.5	5.3 ± 1.1	3.6 ± 1.5
E16	2.3 ± 0.6	Not done	Not done

RESULTS

Expression of Thy1, dlk and oncostatin receptor M β in fetal livers

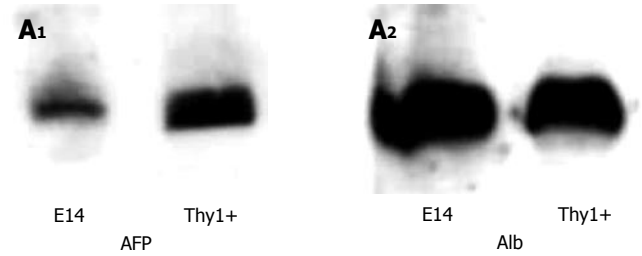
We assessed the expression of oval cell markers, Thy1 and oncostatin receptor M β (OSMRβ), and fetal hepatoblast marker dlk in E13, E14 and E16 fetal livers (Table 1). The expression of all three markers decreased with liver development (Table 1). The largest percentage of Thy1 positive cells was in E13 rat livers (10%), and the percentage of Thy1-positive cells decreased with liver development, so that there were 7.8% Thy1 positive cells in E14 livers, and only 2.3% Thy1 positive cells in E16 livers (Table 1). We did not continue to test the percentages of Thy1 positive cells in later stages, since the peak of hematopoiesis in fetal rat liver was at E17 and E18, and Thy1- positive cells at those stages might be hematopoietic cells rather than hepatoblasts.

We defined the positive populations for both Thy1 and α-fetoprotein in E14 fetal livers. In the E14 total liver population, 4.8% of the cells were positive for both Thy1 and α-fetoprotein, while 3 % were positive for Thy1 only (Table 1). There 2.7% of cells positive for dlk and AFP and 1.6% of cells positive for OSMRβ and AFP in the E14 livers.

Characterization of Thy1- positive cells from E14 fetal livers

We obtained 11 × 10^6 single cell suspension cells from per E14 liver, with a viability > 95% determined by trypan blue exclusion. We selected Thy1- positive cells from the single cell suspensions of E14 liver cells using nanoparticles and magnet selection of Thy1-FITC labeled cells. We determined AFP and albumin expression by Western blots using freshly isolated E14 liver cells and Thy1- selected cells. Thy1-positive cells expressed slightly more AFP than E14 cells, while albumin expression was similar in the two cell populations (Figure 1A). We next determined the percentages of albumin positive cells in the Thy1- selected cells from E14 and E16 livers, using immunocytochemistry staining on cytospun cells. Albumin expression in E14 Thy1- selected cells is shown in Figure 1B. We counted albumin positive cells in 20 fields and found that 25% ± 8% of Thy1+ from E14 livers were albumin positive and 18% ± 9% of Thy1+ cells from E16 livers expressed albumin.

We also determined the expression of oval cell markers c-kit, OSMRβ and CXCR4, liver- enriched transcription factors HNF1α, HNF4α and HNF6, hepatocytic markers AFP, albumin, CK18, and biliary epithelial marker CK19



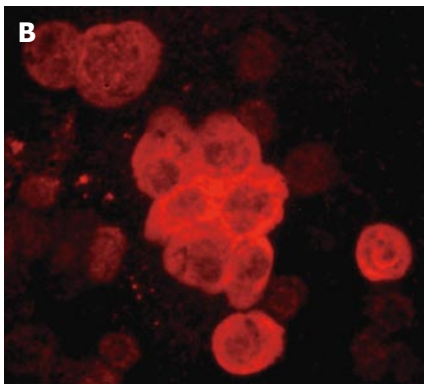


Figure 1 Western blot showing albumin and AFP expression in E14 livers and Thy1 selected cells from rat E14 fetal livers (**A**) and albumin staining on cytospins of Thy1 selected cells from E14 livers (**B**).

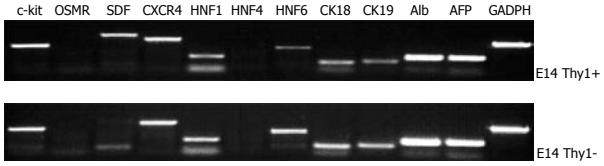


Figure 2 Expression of various oval cell markers, liver-enriched transcription factors and hepatic or bile duct specific markers in Thy1 positive and Thy negative cells from rat E14 fetal livers (RT-PCR).

in Thy1+ and Thy1- cells from E14 livers. We observed that both cell populations were positive for c-kit, CXCR4, HNF1 and HNF6, albumin, AFP, CK18 and CK19, while only the Thy1+ cells were positive for SDF1α, and we could not detect OSMRβ and HNF4 in either cell population (Figure 2).

Culture of Thy1- positive cells isolated from E14 fetal livers

We plated the Thy1-selected cells at clonal densities on collagen type I-coated dishes and in four different media, as described in Materials and Methods. After two weeks, colonies were stained with crystal violet solution and counted. Both medium 2 and medium 3 were beneficial for development of colonies (Table 2). However, the morphology of the cells was not typical of hepatoblasts, but rather of fibroblasts (data not shown). We isolated four clones from two separate experiments of cells grown in medium 3 and on collagen I, and tested them by RT-PCR for hepatic markers. The only hepatic marker detected was CK18 (data not shown).

Since 25% of the Thy1- selected cells expressed albumin, we thought that in the above conditions, the

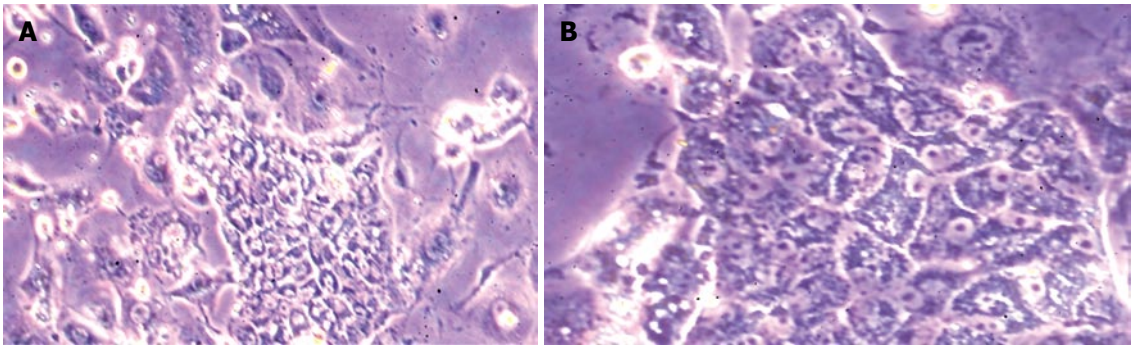


Figure 3 Morphology of colonies of Thy1-selected cells cultured for 1 wk on STO growth-arrested feeder layers. (**A**) X 25; (**B**) X 100.

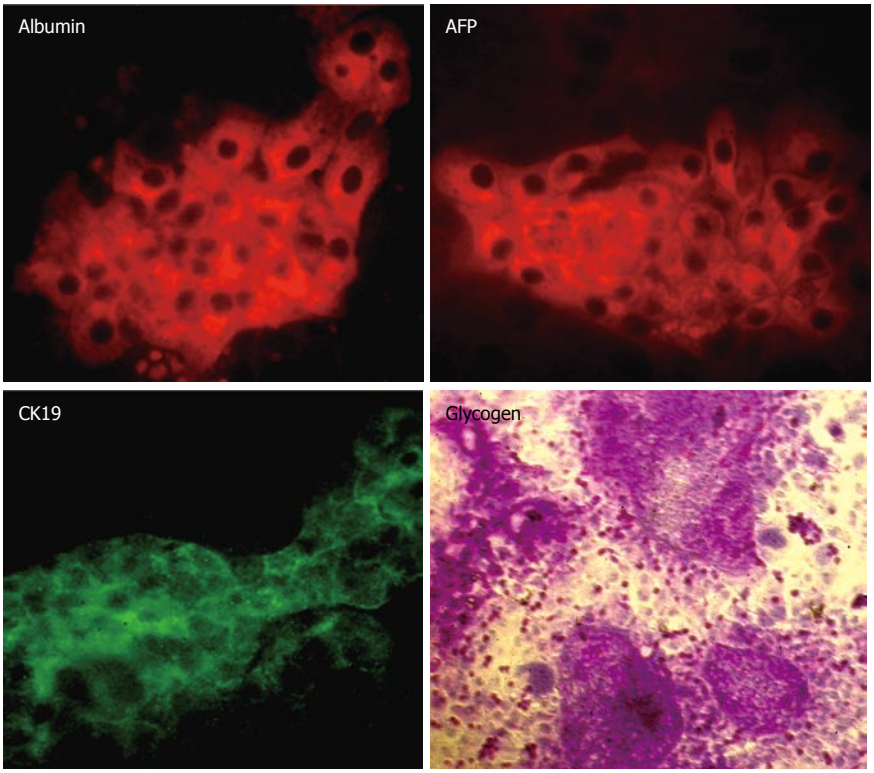


Figure 4 Colonies of Thy1-selected cells cultured for 2 wk on STO growth- arrested -feeder layers and stained with antibodies to albumin, AFP and CK18, and with a PAS detection kit for glycogen. The experiments were repeated three times had similar results.

Table 2 Clonal growth of Thy1- selected cells from E14 livers after 2 wk in various media (mean ± SD)

Media	Colonies (*n*)	Colonies (*n*)
Medium 1	7.3 ± 2	ND
Medium 2	11 ± 6.8	ND
Medium 3	9.5 ± 2	28 ± 8.7
Medium 4	ND	54 ± 1.8

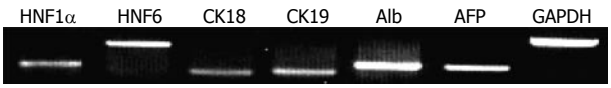


Figure 5 Hepatic and biliary epithelial markers in Thy1 positive selected cells cultured for 2 wk on STO growth- arrested feeder layers (RT-PCR).

albumin-positive cells probably did not survive. Therefore, we plated Thy1-selected cells on collagen type I-coated plates and in DMEM with 100 mL/L FCS, at a density of 500 000 cells/well in 6-well plates. After one and two days in culture, typical fetal hepatoblast colonies were observed and stained positive for albumin (data not shown). However, the colonies did not survive for longer periods, but peeled off the plates. Since hepatic fetal cells could grow on feeder layers[7], we plated Thy1- selected cells on STO feeders and in serum free-medium. The cells survived very well under these conditions for two weeks, and formed typical fetal hepatoblast colonies (Figure 3). These colonies were positive for albumin, AFP, CK18 and glycogen (Figure 4). We determined that 0.6% ± 0.17% of the total Thy1 positively-plated cells formed albumin-positive colonies. However, there were numerous other colonies on the plates, with fibroblast morphology similar to the colonies obtained when the cells were cultured on collagen I and in media 1-4 (data not shown).

We examined the expression of mRNA for hepatocytic markers in two weeks- old cultures of Thy1-selected cells grown on STO feeders. We found that there was expression of liver enriched-transcription factors HNF1α and HNF6, as well as hepatic markers albumin, AFP, CK18 and biliary marker CK19 (Figure 5). The expression

of OSMRβ was very weak in the colonies derived from Thy1 positive cells, while transcription factor HNF4α was not detected (data not shown).

DISCUSSION

In the present study, early rat fetal livers contained Thy1-positive cells, and the percentage of Thy1- positive cells decreased between E13 and E16. E14 fetal livers contained about 8% Thy1-positive cells, of which 61% were positive for AFP and 25% were positive for albumin. The Thy1-positive cells expressed oval cell markers c-kit and CXCR4, liver enriched-transcription factors HNF1α and HNF6, hepatocytic markers albumin, α fetoprotein and CK18, and biliary marker CK19. We were able to culture Thy1-selected cells from E14 livers for two weeks on STO feeder layers, and observed several types of colonies, both mesenchymal and hepatic colonies.

In the last few years, hepatic stem cells from various sources, both fetal and adult livers, have been studied extensively. Methods used to isolate the cells from murine and rat fetal livers include FACS sorting using several positive and negative markers[6,7]. When the isolated cells are cultured, they form colonies that contain both hepatocytes and bile duct epithelial cells.

We hypothesized that in addition to the fetal hepatoblasts, which could differentiate into hepatocytes and bile duct epithelial cells during fetal liver development, fetal liver might contain additional stem cells, the "oval" cells. We may surmise that cells positive for oval cell markers Thy1 and AFP might be more numerous in the early stages of fetal liver development and decrease in later stages, so that the oval cell numbers in the adult livers are very few, and in fact difficult to detect unless oval cells proliferate due to carcinogenic injuries.

Thy1 has been shown to be present on rat oval cells, one kind of the putative liver stem cells in the adult liver[9]. Thy1 (CD90) is a cell surface molecule, present on hematopoietic stem cells, subsets of T cells, bone marrow cells, fibroblasts and cells in the brain[22,23]. Thy1 may be a receptor for an unknown ligand, and may play a role in cellular recognition and adhesion [20,21].

Adult rat liver oval cells, in addition to Thy1, expressing AFP, cytokeratin 19 and GGT, are negative for hepatic stellate cell marker, desmin[9]. Other markers for oval cells are c-kit, oncostatin M receptor β and CXCR4[10,14]. Recent studies have shown that Thy1 is present not only on adult rat oval cells, but also in rat E16 fetal liver, and a small percent of Thy1 positive cells also express hepatocytic marker CK18[12], suggesting that the Thy1 + CK18 + are hepatic stem cells. The same group isolated Thy1 positive cells from E16, E18, E20 and E22, and found that the cells are also positive for albumin, AFP and CK18[13]. Long-term cultures (one month) of human fetal hepatocytes also express Thy1, as well as other oval cell and hematopoietic markers such as CD34 and OV-6. In addition, these cells assume a blast-like morphology and express hepatoblast markers AFP, CK19 and CK18[24].

Rat fetal liver rudiment appears at E10, E13 and E14, there are still numerous bipotential hepatic stem cells, though there are also cells committed towards the hepatocyte and biliary epithelial cell lineage[25]. Since the rat fetal liver is both a hepatic and a hematopoietic organ, we tested the expression of Thy1, a hematopoietic marker, before the peak of hematopoiesis in fetal liver, which occurred at E17 and lasted until birth. We found that there were Thy1 positive cells in E13 livers, and the percentage of Thy1 positive cells decreased between E13 and E16.

We chose E14 fetal livers as the ideal stage for isolating oval cells, since the livers contain a large number of cells, and there are many Thy1+ AFP+ cells. Moreover, we observed that Thy1 cells selected from E14 livers expressed numerous hepatic and biliary epithelial markers and liver-enriched transcription factors, as well as oval cell markers c-kit and CXCR4, and OSMRβ detected by FACS analysis, but not by RT-PCR[10]. However, we did detect OSMRβ in colonies of Thy1 positive cells, suggesting that its expression is very weak in the original cells. The Thy1-selected cells were not homogenous and contained both oval cells (Thy1+ AFP+) and mesenchymal cells, (Thy1+ AFP-). When we cultured the cells on collagen I and in various media containing serum, we observed only mesenchymal cell colonies and some epithelial colonies that were positive for CK18. However, when we cultured the Thy1 positive cells on STO feeder layers, we detected formation of hepatic colonies expressing albumin and AFP. The original Thy1+ cells and colonies derived from Thy positive cells were negative for HNF4. It was reported that oval cells are negative for HNF4, and only start expressing this transcription factor when they differentiate into hepatocytes[26], suggesting that fetal oval cells may not undergo complete differentiation into hepatocytes under our culture conditions.

In mice, contrary to rat and human livers, Thy1 appears to identify only mesenchymal cells both in adult and in fetal liver[27,28]. Thy1 positive cells isolated from murine fetal livers have been shown to support differentiation of CD49f positive cells to hepatocytes[28]. The Thy1 positive cells comprise three different populations, one positive for α-smooth muscle actin, another positive for desmin, both markers of hepatic stellate cells, and a third population negative for both[28]. The murine Thy1 positive cells are negative for AFP, cytokeratin 19 and albumin[28].

In conclusion, Thy1 identifies putative oval cells during early fetal liver development, but is also present on other cells such as mesenchymal cells.

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BASIC RESEARCH

Curcumin-attenuated trinitrobenzene sulphonic acid induces chronic colitis by inhibiting expression of cyclooxygenase-2

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Abstract

AIM: To explore the possible mechanisms of curcumin in rat colitis induced by trinitrobenzene sulfonic (TNBS) acid.

METHODS: Rats with TNBS acid-induced colitis were treated with curcumin (30 mg/kg or 60 mg/kg per day ip). Changes of body weight and histological scores as well as survival rate were evaluated. Leukocyte infiltration was detected by myeloperoxidase (MPO) activity assay. The expression of cyclooxygenase-2 (COX-2) was detected by RT-PCR and Western blot. Inflammation cytokines were determined by RT-PCR. Local concentration of prostaglandin E_2 (PGE_2) in colon mucosa was determined by ELISA.

RESULTS: Curcumin improved survival rate and histological image, decreased the macroscopic scores and MPO activity. Also curcumin reduced the expression of COX-2 and inflammation cytokines. In addition, treatment with curcumin increased the PGE_2 level.

CONCLUSION: Curcumin has therapeutic effects on TNBS acid-induced colitis, the mechanisms seem to be related to COX-2 inhibition and PGE_2 improvement.

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Key words: Colitis; Curcumin; Cyclooxygenase-2; PGE_2; TNBS acid

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INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by chronic recurrent ulceration of the bowel. Frontline drugs that are currently used for the treatment of IBD include derivatives of 5-aminosalicylic acid (5-ASA), glucocorticoid and immunosuppressives. Novel agents such as monoclonal antibodies against tumor necrosis factor alpha (TNF-α) are now commercially available[1,2]. However, these agents have varying efficacy and are expensive. Consequently, there is a need for alternative agents that may be equally or more effective and cheaper. Curcumin is the prominent yellow pigment in turmeric, a widely used spice and food colouring agent with anti-inflammatory and anti-cancer properties[3]. Mechanisms by which curcumin exerts its pharmacological effects are thought to involve antioxidation[4], inhibition of kinases, interference with the activity of transcription factors such as NF-κB and AP-1[5]. Cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) are inhibited by curcumin through NF-κB dependent or independent pathway[6,7].

In IBD, increased COX-2 mRNA and protein have been found in inflamed areas, and most of the PGs are produced by the COX-2 pathway. Hence, inhibition of excessive COX-2 activity and PGE_2 production may result in suppression of the inflammatory response. Preventive and therapeutic mechanisms of curcumin are to inhibit the activation of NF-κB and reduce the activity of p38 MAPK[8-11]. But whether treatment of colitis with curcumin reduces COX-2 and PGE_2 remains unknown. In the present study, we investigated the effect of curcumin on body weight changes, the expression of COX-2, the local concentration of PGE_2 in colon mucosa and the changes of inflammatory genes in rats with colitis induced by trinitrobenzene sulphonic (TNBS) acid.

MATERIALS AND METHODS

Experimental animals

Specific pathogen-free Sprague-Dawley rats weighing 200-220 g were obtained from Laboratory Animal Center of Wuhan University (Wuhan, China) and kept at constant temperature (23 ± 2℃) and humidity (50%-70%) in a 12 h light and dark cycle. The rats were allowed to adapt to our laboratory environment for one week before experiment with free access to standard rodent chow and tap water.

Induction of colitis

Colitis was induced by TNBS acid using the modified method described by Morris *et al*[12]. Briefly, rats were fasted the day before colitis induction. Each rat was anesthetized with ether, and 100 mg/kg of TNBS acid (Sigma, St. Louis, MO, 100 g/L TNBS acid in 500 mL/L ethanol, total volume 1.0 mL) was then instilled via a rubber catheter inserted 8 cm into the colon *via* the anus. The rubber catheter was modified with numerous holes in the last 4 cm of its length. The instillation procedure required only a few seconds and the rats were maintained in a vertical position for 3 min to prevent solution leakage. Control mice received 500 mL/L ethanol of the same volume using the same technique. All the rats were checked daily for behavior and body mass. The experiments were approved by the Institution's Animal Care and Use Committee.

Treatment protocols

To investigate the therapeutic and dosage effects of curcumin(≥ 95%, Sigma), rats were randomly divided into four groups: control group(n = 15, receiving ethanol only and no treatment), TNBS acid group(n = 15, receiving TNBS acid and no treatment), curL group(n = 15, receiving 30 mg/kg curcumin per day ip) and curH group(n = 15, receiving curcumin 60 mg/kg per day ip). On d 1, the rats were fasted, colitis was induced and treatment was begun on d 2. Con and TNBS acid groups received the same volume of 5% ethanol (vehicle of curcumin) everyday until the end of the experiment. Treated groups received curcumin just after colitis was induced for two weeks until the rats were sacrificed.

Assessment of severity of colitis

After rapid removal of the colon, specimens were flushed with ice cold PBS, cut open and photographed. The photographs of the colonic specimens were then scored by a blinded observer unaware of the treatment. Scores were assessed by using the following damage scoring system[12]: 0: no damage; 1: localized hyperaemia without ulcers; 2: linear ulcers with no significant inflammation; 3: linear ulcer with inflammation at one site; 4: two or more sites of ulceration and/or inflammation; 5: two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending more than 1 cm along the colon. For histological analysis, tissues were fixed in 100 g/L paraformaldehyde in phosphate-buffered saline, and paraffin-embedded tissue sections were stained with hematoxylin and eosin (HE) using the standard techniques.

Assessment of leukocyte infiltration

Myeloperoxidase (MPO) activity was assessed as a marker of neutrophil infiltration. Rat colon samples were snap-frozen in liquid nitrogen and stored at -80℃ before MPO activity assessment. The tissue was thawed, weighed and homogenized in PBS, the homogenate was centrifuged and the pellet was again homogenized in PBS containing 5 g/L hexadecyl-trimethylammonium bromide (HETAB) and 10 mmol/L EDTA. This homogenate was subjected to three cycles of freezing/thawing and brief sonication. A sample of homogenates was added to reaction volume (containing 80 mmol/L PBS, pH 5.4, 5 g/L HETAB and 1.6 mmol/L 3, 3', 5, 5'-tetramethylbenzidine). The mixture was incubated at 37℃ for 5 min and the reaction was started by the addition of H_2O_2. The complete reaction mixture was incubated for exactly 3 min at 37℃ and terminated by the sequential addition of catalase and sodium acetate. The changes in absorbance at 655 nm were measured with a spectrophotometer.

Semi-quantitative RT-PCR

For RT-PCR, rat colon samples were snap-frozen in liquid nitrogen and stored at -80℃ before ribonucleic acid (RNA) preparation. Total RNA was isolated using the TRIzol method (Life Technologies, Canada). Concentration of the RNA was detected from A_{260}, and the integrity of the RNA was verified by electrophoresis on formaldehyde gels. Total RNA was reverse-transcribed into complementary deoxyribonucleic acids (cDNAs) using a first strand cDNA synthesis kit (Fermentas, Life Sciences).The resultant cDNAs were subjected to PCR for measurement of messenger RNAs (mRNAs). The PCR products were subjected to agarose gel electrophoresis and the abundance of each mRNA was normalized to that of GAPDH. The sequences of all primers used in this project are as flowing:

GAPDH: sense: 5'ATGGGTGTGAACCACGAGAAA-3',
anti-sense: 5'GGATACATTGGGGGTAGGAA-3'(330 bp);

COX-2: sense: 5'-TACAAGCAGTGGCAAAGGC-3',
anti-sense: 5'-CAGTATTGAGGAGAACAGATGGG-3'(304 bp);

TNF-α: sense: 5'-TACTGAACTTCGGGGTGATTGGTCC-3',
anti-sense: 5'-CAGCCTTGTCCCTTGAAGAGAACC-3'(295 bp);

IFN- sense:5'AGCCTAGAAAGTCTGAAGAAC-3',
anti-sense:5'ACCGACTCCTTTTCCGCTTCCT-3'(387 bp)

iNOS: sense:5'-TGAAGCACATGCAGAAATGAGTACCG-3',
anti-sense: 5'-CCGTCAGAGGTAACTGTTTACACG-3' (464 bp)

Western blot analysis

Western blot analysis was performed by standard methods. Briefly, frozen tissue samples were homogenized in Tris-HCl buffer containing a cocktail of protease inhibitors and insoluble materials removed by centrifugation at 4℃. The solubilized lysates were resolved by sodium dodecyl sulfate (SDS)-PAGE electrophoresis under reducing conditions at a concentration of 50 μg protein of each sample per lane. Nitrocellulose membranes were incubated overnight with rabbit anti-serum directed against COX-2 (Santa Cruz Biotechnology). Immunodetection with secondary peroxidase-conjugated antibody and chemiluminescence was performed according to the manufacturer's protocol (Santa Cruz Biotechnology). Density of the products was quantified.

Enzyme-link immunosorbent analysis

Colonic mucosal samples kept at -80℃ were weighed and homogenized on ice in 1 mL Tris-HCl buffer containing 5.6 mmol/L indomethacin (pH 7.4), the homogenization

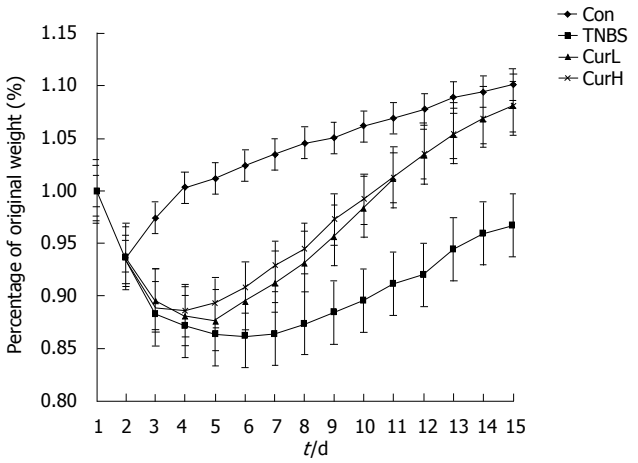


Figure 1 Body mass changes in rats with TNBS acid-induced colitis. Each bar represents mean ± SE. $P < 0.05$ *vs* TNBS acid.

was vortexed thoroughly for at least 2 min. After centrifugation, the concentration of PGE_2 in supernatants was immediately determined with competitive ELISA kits (R&D), according to the manufacturer's instructions. PGE_2 levels were normalized to microgram of protein. The detection limit of PGE_2 was 39 ng/L.

Statistical analysis

Data were expressed as mean ± SE. The statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered statistically significant. In the experiment involving histology, the figures shown are representative of at least three experiments performed on different days.

RESULTS

Body mass

Administration of TNBS acid caused a dramatically decrease in body mass of all groups. Most of them decreased more than 30 g after 2 d (data not shown). Con group showed transient and slight loss of body weight (less than 10 g, data not shown) and recovered quickly. From d 3 it began to show significant difference from TNBS acid group ($P < 0.05$, Figure 1). At the end of the experiment, except for TNBS acid group, body weight of the other groups all reached the initial level. No difference was observed among control, curL and curH groups at the end of the experiment.

Macroscopic scores and histological images

TNBS acid-induced colitis was characterized by thick and stiff colonic wall due to edema, chemical erosion or fibriform-proliferation. Ulcers were scattered along the colon or linked with each other, bleeding or redness was observed in whole or partial colon. Necrosis of epithelium, distortion of crypts, destruction of glands and infiltration of inflammatory cells were observed. The macroscopic scores in curL and curH groups were significantly lower than those in TNBS acid group ($P < 0.01$ Figure 2). Treatment with curcumin decreased edema. Ulcers in curcumin-

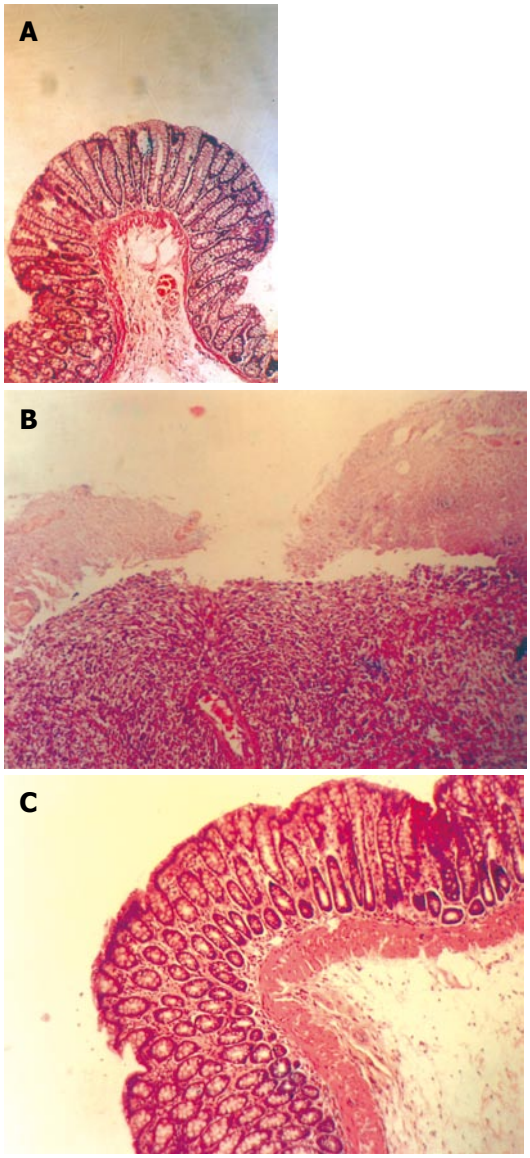


Figure 2 Effects of curcumin on histological scores of rats with TNBS acid-induced colitis in control group (**A**), TNBS acid group (**B**), and cur group (**C**). (HE staining, original magnification: × 200).

treated groups were much smaller and superficial, most of the ulcers were healed and granulation or fibroplasias could be seen. Histological analysis showed that curcumin treatment decreased necrosis of epithelium and the infiltration of inflammatory cells and granulation can be seen.

Leukocyte involvements

Myeloperoxidase activity was significantly ($P < 0.01$) increased in TNBS acid-treated animals compared with control group (101 020 ± 17 170 nkat/g and 249 382 ±43 342 nkat/g tissue protein, respectively, Table 1), which was consistent with the histological findings. Treatment with curcumin (30 mg/kg or 60 mg/kg per day) significantly reduced the degree of polymorphonuclear and neutrophil infiltration in rats with TNBS acid-induced colitis.

COX-2 and PGE_2 expression

Weak expression of COX-2 mRNA could be detected in normal colon (Figure 3). After colitis was induced by

Table 1 Concentrations of PGE_2 , activities of MPO in colon tissue and mRNA in inflammatory response genes (mean ± SE)

	Tissue	Protein	mRNA		
	PGE_2 (ng/g)	MPO (knat/g)	TNF-α	IFN-γ	iNOS
Con 15	176.3 ± 29.2[c]	101 020 ± 17 170[d]	0.0795 ± 0.0096[d]	0.0284 ± 0.0150[d]	0.8304 ± 0.1180[d]
TNBS 8	78.5 ± 13.2[a]	249 382 ± 43 342[b]	0.8861 ± 0.0533[b]	1.0212 ± 0.0687[b]	6.5690 ± 0.9450[b]
CurL 12	190.2 ± 17.0[c]	121 691 ± 23 171[ad]	0.1967 ± 0.0365[bd]	0.0565 ± 0.0177[ad]	1.8638 ± 0.3347[bd]
CurH 13	215.5 ± 20.4[ac]	122 191 ± 21 171[ad]	0.1727 ± 0.0348[bd]	0.0518 ± 0.0057[d]	1.7250 ± 0.3026[bd]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control; [c]$P < 0.05$, [d]$P < 0.01$ *vs* TNBS acid (Dunnett's *t* test)

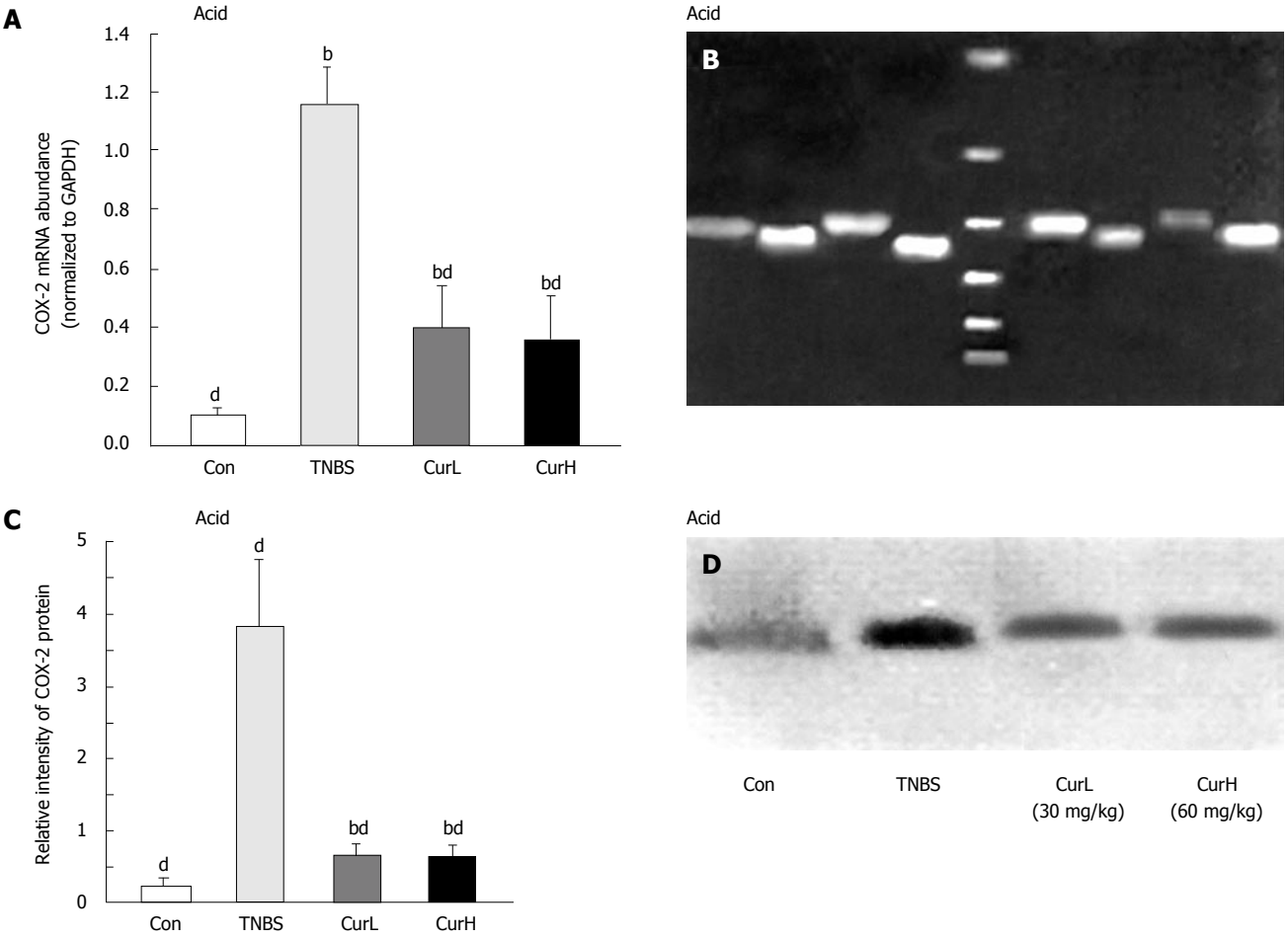


Figure 3 COX-2 expression in rats with TNBS acid-induced colitis. **A**: COX-2 mRNA; **B**: COX-2 products; **C** and **D**: COX-2 protein (M_r72 000) (mean ± SE, Dunnett's *t* test). [b]$P < 0.01$ *vs* control; [d]$P < 0.01$ *vs* TNBS acid.

TNBS acid, its expression increased by more than 10-fold. Treatment with curcumin decreased its expression by 70%, but was still higher than normal ($P < 0.05$). Western blot analysis showed the similar changes of protein in colon mucosa (Figure 3). After colitis was induced, PGE_2 decreased and its expression was increased after curcumin treatment.

Expression of inflammatory mediator genes

Abundance of IFN-γ, TNF-α and iNOS mRNA in TNBS acid group was significantly elevated compared with control group. Curcumin treatment suppressed the increased mRNA efficiently, but its level was still higher than normal (Table 1).

DISCUSSION

Treatment with curcumin (30 mg/kg or 60 mg/kg per day) reduced the severity and extension of damage induced by TNBS acid, decreased the extent of colitis and increased the survival rate and the incidence of adhesions. The rats treated with curcumin had improved histological image and less neutrophil infiltration in colon mucosa. The expression of COX-2 increased in acute and chronic colitis, suggesting that over-expression of COX-2 may result in neoplasia[13-17]. Curcumin exerts its antitumor and anti-inflammation effects by down-regulating COX-2 directly or indirectly[18]. Some reports indicate that selective COX-2 inhibitors exacerbate colitis[19-21], but others indicate that

they have therapeutic effects on colitis[22-26]. In our experiment, the expression of COX-2 increased in colon following the induction of colitis, suggesting that high expression of COX-2 in the colon is associated with high MPO activity and cytokine production. Curcumin decreased the level of COX-2, indicating that decreasing the expression of COX-2 may benefit to colitis.

PGE_2, an inflammatory mediator, participates in epithelium repairing and exerts anti-inflammation effects including suppression of neutrophil function or prevention of mast cell degranulation. Recently, PGE_2 has been implicated in the inhibition of the production of IFN-γ, IL-2 and IL-12[27]. It was reported that in early or acute inflammation phase the expression of PGE_2 is usually upregulated, resulting in inflammatory manifestations, such as redness, edema, pyrexia and ache[15,28]. Treatment with curcumin increased the expression of PGE_2 though its limiting enzyme COX-2 decreased, indicating that curcumin can change the expression spectrum of PGs in colitis.

TNBS acid-induced chronic intestinal inflammation mimics human Crohn's disease, showing high IFN-γ, TNF-α and iNOS, but low levels of IL-4 and IL-5[19,29]. Treatment with curcumin decreased IFN-γ, TNF-α and iNOS distinctly in our study, which is consistent with the reports of Ukil *et al*[11] and Sugimoto *et al*[9]. Since PGE_2 is capable of inhibiting Th1 cytokines, the decreased IFN-γ, TNF-α and iNOS in this study maybe due to PGE_2. Although curcumin decreased the expression of inflammatory response genes, but not decrease it to the normal level, suggesting that curcumin is not able to completely eliminate colitis.

Curcumin (30 mg/kg or 60 mg/kg per day) did not show any difference in the treatment of colitis induced by TNBS acid. Curcumin is also a chemopreventive drug for many tumors[18]. IBD patients are at risk of developing dysplasia and neoplasia. Phase I clinical trial of curcumin indicated that 8 g/d for 3 mo does not show any toxicity[30], suggesting that long-term administration of curcumin in IBD patients is not only safe but also improves colitis and prevents development of colorectal cancer.

In conclusion, curcumin attenuates TNBS acid-induced chronic colitis by decreasing the expression of COX-2 and increasing PGE_2. Curcumin can down regulate the expression of inflammatory genes in colitis induced by TNBS acid and is a safe and therapeutic agent for IBD patients

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BASIC RESEARCH

Expression of macrophage inflammatory protein-1α in Kupffer cells following liver ischemia or reperfusion injury in rats

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Abstract

AIM: To explore the expression of macrophage inflammatory protein-1α (MIP-1α) in Kupffer cells (KCs) following liver ischemia/reperfusion injury IRI in rats.

METHODS: Forty male SD rats were divided randomly into five groups. A model of partial warm ischemia/reperfusion injury in the rat liver was established. KCs were isolated and incubated one hour, six hours, 12 h, and 24 h after the reperfusion. Tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) in the supernatants were measured by ELISA. MIP-1α in KCs was detected by immunocytochemical and RT-PCR.

RESULTS: No or few MIP-1α protein and mRNA were expressed in the KCs of the control group. Its expression in the IRI group had a significant increase after the reperfusion ($P < 0.05$), which was contrary to the control group.

CONCLUSION: The active behavior of the MIP-1α gene in KCs following liver ischemia/reperfusion injury is assumed to be one of the major causes for the hepatic ischemia/reperfusion injury.

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Key words: Liver; Ischemia/reperfusion; Kupffer cell; Macrophage inflammatory protein-1α

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INTRODUCTION

Hepatic ischemia/reperfusion injury (IRI) is a common pathological process of traumatic surgical diseases in the liver, such as severe liver trauma, extensive hepatic lobus excision, liver transplantation, shock and infection[1]. It can cause a series of injuries on metabolism, structure, and function in hepatic tissues and cells, and even liver function failure. It is also one of the major factors influencing the prognosis, operative success and survival of patients. The mechanisms of liver injury caused by ischemia/reperfusion resemble those observed in other organs that are transiently deprived of oxygen. These mechanisms involve a series of events, including Kupffer cell (KCs) activation, cytokine release, neutrophil activation, increased expression of adhesion molecules, sinusoidal endothelial cell death, and hepatocyte injury[2]. Among these, the activation and the release of the excessive quantities of cytokine in the Kupffer cells play a major role.

Chemokines are members of a large and expanding family of proteins that interfere with normal (leukocyte homing, hematopoiesis, angiogenesis) and pathological (inflammation, HIV-1 infection, atherosclerosis) processes[3-7]. Depending on the positioning of the cysteine residues, chemokines are classified as C, CC, CXC and CX_3C chemokines. MIP-1α is from the CC (cysteinecysteine) chemokine subfamily. These soluble factors are chemotactic for specific types of leukocyte populations and are involved in the regulation of cell-mediated immunity[8]. MIP-1α is produced by monocytes, macrophages, lymphocytes, and other cell types[9]. CCR1, CCR4, and CCR5 are the known receptors that bind to MIP-1α[10]. In addition, it has been demonstrated that these CC chemokines can induce mononuclear macrophage to secrete TNF-α and IL-1β[11]. For all the significant roles that these CC chemokines play in immune and inflammatory responses, their role in the mechanism of hepatic ischemia/reperfusion injury is not well understood. Therefore, we established a model of rat hepatic ischemia/reperfusion injury at the room temperature, isolated the Kupffer cells at different times, that is, 0 h, 1 h, 6 h, 12 h and 24 h after the onset of reperfusion, to explore the expressions of MIP-1α protein and mRNA in the Kupffer cells following liver ischemia/reperfusion injury.

MATERIALS AND METHODS

Materials

Type Ⅳ collagenas and percoll were purchased from

Sigma, Inc, (USA). Triozol was from Invitrogen, Inc, (USA). RNA PCR Kit and DNA Maker were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (China). Lysozyme, MIP-1α polyclonal antibody and SABC kit were purchased from Wuhan Borsd Biological Engineering Co., Ltd. (China). TNF-α and IL-1β ELISA kit were obtained from Shanghai Sengxiong Biotech Industry Co., Ltd. (China).

Animals

Male Sprague Dawley (SD) rats weighing 250-300 g were used in the study. They were obtained from the Experimental Animal Center of the Xi'an Jiaotong University. All animals were housed in a macroion cage in rooms maintained at a temperature of 22-24℃ using a 12/12-h light/dark cycle. The animals were given a standard rat chow and fasted overnight before the experiment with water allowed ad libitum. Care was provided in accordance with the procedure outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication No.85-23, revised 1996). The study was approved by the subcommittee on research animal care at our institution.

Hepatic ischemia/reperfusion injury model in rats

Forty-eight SD rats were divided randomly into six groups: the sham operation group (control group) and the ischemia/reperfusion injury Ⅰ-Ⅴ group (IRI Ⅰ-Ⅴ group). The trial rats were starved for 12 h but were allowed to drink water. A model of the partial warm hepatic ischemia/reperfusion injury was established at room temperature by the Nauta *et al* method[12]. In the experiment, the rats were anesthetized with aether. The liver was exposed by a midline incision before operation. The left lateral and median hepatic lobes were occluded with a microvascular clamp for 45 min. After 45 min of ischemia, reperfusion was induced by removing the vascular clamp. 0 h, 1 h, 6 h, 12 h and 24 h after the reperfusion, the rats were killed to isolate the Kupffer cells at the same time. The sham control rats underwent the same treatment, but without vascular occlusion.

Kupffer cells isolation

With the reference to the collagenase perfusion technique described by Knittel *et al*[13], the Kupffer cells were isolated by an improved method. In the experiment, the liver was perfused for 30 min with D-Hank's containing 0.01% EDTA and removed to be smashed by scissors. The resulted stuff was digested for 45 min in a solution of 0.5 g/L type Ⅳ collagenase and was filtered through 200-mesh strainer. The filtrate was washed thoroughly and suspended in 3mL PBS, layered on 3 mL 25% percoll solution and 3 mL 50% percoll solution, centrifuged at 2500 r/min for 25 min to separate the cells. The cells between the layers of 25% percoll solution and 50% percoll solution were carefully extracted. The freshly isolated cells were suspended in DMEM medium supplemented with 25 mM HEPES, 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mmol/L). Cell suspensions (4 mL, 8×10^6 cells) were plated on 60-mm culture dishes and maintained in an incubator at 37℃ in a humidified atmosphere of 90% air-100 mL/L CO_2 for 1 h. The supernatants were discarded and the adherent cells were incubated for 4 h. The cellular components of adherent cells were examined by Lysozyme immunohistochemical staining to determine the percentage of Kupffer cells. The percentage of Kupffer cells in the adherent cells was over 90% (Figure 1). The cells were counted after the trypan blue exclusion. The viability of the cells was over 90% in the adherent cells. The Kupffer cells and supernatants were collected in all the groups and stored in refrigeration at -70℃ for subsequent analysis.

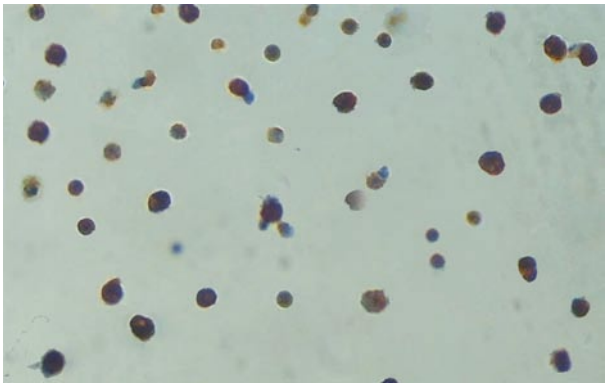


Figure 1 Lysozyme immunohistochemical staining of the Kupffer cells. The cytoplasms of the Kupffer cells were stained brown. The percentage of Kupffer cells in the adherent cells was over 90%.

Expression of TNF-α and IL-1β in supernatants

TNF-α and IL-1β in the supernatants of primary cultures of rat Kupffer cells were measured by TNF-α and IL-1β ELISA kits. The concentrations of TNF-α and IL-1 were estimated based on the absorbance read by an ELISA reader (EL800) at 450 nm.

Immunocytochemiscal detection of MIP-1α protein

Immunocytochemiscal staining of Kupffer cells was performed with the strept-avidin-biotin-peroxidase complex (SABC) method.[14] Kupffer cells adhered to glass slides were fixed with aether and ethanol. They were soaked in 30% hydrogen and pure methanol (1:50) for 30 min at room temperature. After incubation in normal goat serum blocking solution, the glass slides were incubated in rabbit anti-rat MIP-1α polyclonal antibody (1:200) at 4℃ overnight. PBS takes the place of MIP-1α antibody as the negative staining. Then the glass slides were incubated in biotined goat anti-rabbit IgG for 20 min at 20℃. Finally, the values of the mean optical density were measured under the same magnification using an image analysis system (original magnification × 400).

Determination of MIP-1α mRNA by semiquantitative RT-PCR

Total RNA was isolated from rat Kupffer cells using Trizol reagent. One microgram of RNA was reverse-transcribed to complement DNA using RNA PCR Kit according to the manufacturer's instructions. The PCR primers were synthesized on the basis of Gen-Bank data. The primers were chemically synthesized using DNA

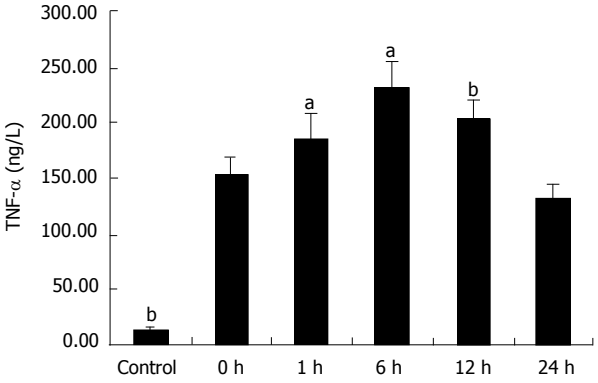


Figure 2 Comparison of TNF-α production in supernatant of Kupffer cells. (n = 8, mean ± SE, $^aP < 0.05$ compared with the next group, $^bP < 0.01$ compared with the next group.)

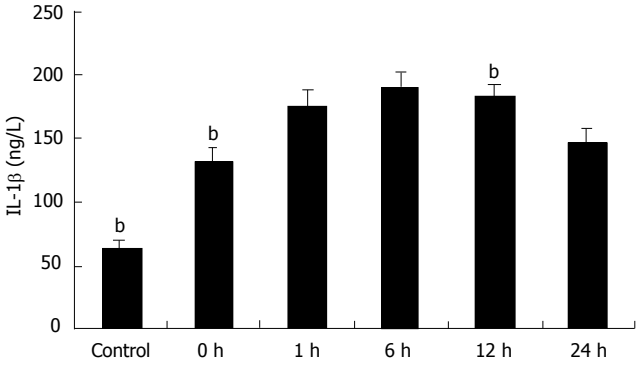


Figure 3 Comparison of IL-1β production in supernatant of Kupffer cells. (n = 8, mean ± SE, $^bP < 0.01$ compared with the next group.)

synthesizer (Tiangen Biotech Co., Ltd, Beijing China). Their sequences were 5'-TTTGAGACCAGCAGCCTTTG-3', 5'-GAAGAGTCCCTGGATGTGGC-3' for MIP-1α and 5'- CATAGACAAGATGGTGAAGG -3', 5'-TCCACAGTCTTCTGAGTGGC -3' for GAPDH. All the PCR reactions had an initial denaturation step at 94℃ for 3 min, and a final extension at 72℃ for 5 min using PTC-100 (MJ Research, Inc. USA.). The PCR amplification cycling conditions were 94℃ 30 s, 52℃ 30 s, 72℃ 30 s, 40 cycles for MIP-1α; 94℃ 30 s, 53℃ 30 s, 72℃ 30 s, 35 cycles for GAPDH. Following RT-PCR, 5 μL samples of amplified products were resolved by electrophoresis in 1.5% agarose gel, and stained with ethidium bromide. The intensity of each PCR product was semiquantitatively evaluated using the labworks analysis software (UVP, Inc, Upland, CA, USA.).

Statistical analysis

Results were expressed as mean ± SE. Statistical calculations were made using the SPSS10.0 software package. One-factor analysis of variance was applied to determine whether the differences among the three groups were statistically significant. In all the cases, P values lower than 0.05 were considered to be statistically significant.

RESULTS

Measurement of levels of TNF-α, IL-1β in supernatants

The cytokines released from the Kupffer cell were studied by ELISA. As shown in Figure 2, the concentration of TNF-α in the supernatant of primary cultures of rat Kupffer cells in the control group was 13.89 ± 2.04 ng/L and it increased at least ten times in IRI group I ($P < 0.01$). Following reperfusion injury stimulation, the TNF-α level was increased with time, and reached the maximum (230.6 ± 26.3 ng/L) 6 h after the stimulation ($P < 0.01$). Even 24 h after the reperfusion, the concentration of TNF-α (133.68 ± 12.15 ng/L) was still significantly high compared with that in the control group ($P < 0.01$). A comparison of IL-1β production in supernatant of Kupffer cells is shown in Figure 3. The concentration of IL-1β in the control group was 64.65 ± 4.63 ng/L and significantly increased with time, reaching its maximum (189.8 ± 13.13 ng/L) 6 h after the reperfusion ($P < 0.01$), and slightly declined (146.30 ± 11.90 ng/L) 24 h after the following reperfusion.

Measurement of the levels of MIP-1α protein in Kupffer cells

Figure 4 shows the immunohistochemical staining of the Kupffer cells that were isolated 12 h after the reperfusion of rat liver. The cytoplasms of these Kupffer cells were stained brown. On the contrary, in the control group, IRI group I and II, the Kupffer cells were not or weakly brown stained. We used an image analysis system to measure the values of the mean optical density of the Kupffer cells in all the groups and found the levels of MIP-1α protein in Kupffer cells had a significant increase at 6 h, 12 h and 24 h intervals ($P < 0.01$), which was contrary to the control group (Figure 5).

Expression of MIP-1α mRNA in Kupffer cells

PCR products were electrophoresed on agarosegels and photographed (Figure 6). The track numbered 1 is for the control group and the tracks numbered 2-6 are for the ischemia/reperfusion injury I-V group. The track marked M is for DNA maker. Quantitative data of MIP-1α mRNA levels in Kupffer cells were represented by the ratio of relative absorbance and expressed as mean ± SD (Figure 7). It showed that the Kupffer cells in the control group had low but detectable levels of MIP-1α mRNA. There was no statistical significance between the control group and the ischemia/reperfusion injury I group ($P > 0.05$). The MIP-1α mRNA level in Kupffer cells significantly increased with time, reaching its maximum 6 h after the reperfusion injury ($P < 0.01$), and slightly declined at 24 h interval but was still much higher than that in the control group ($P < 0.01$).

DISCUSSION

Hepatic ischemia/reperfusion injury is one of the major complications of liver resection surgery, transplantation, and hypovolemic shock[1,15]. The detailed biochemical mechanisms of liver injury caused by ischemia/reperfusion are complex and not well known till now. Several animal

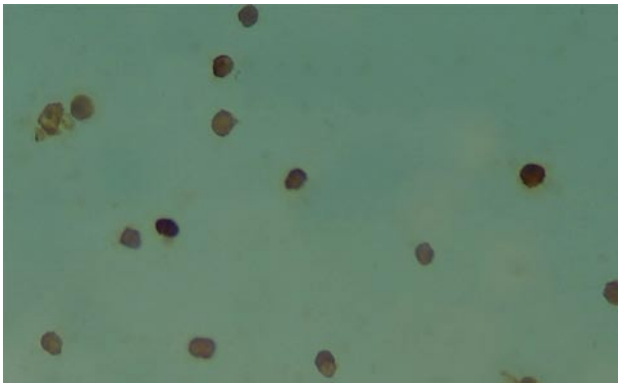


Figure 4 Immunohistochemical staining of MIP-1α in Kupffer cells in IRI group at 12 h's interval SP × 400.

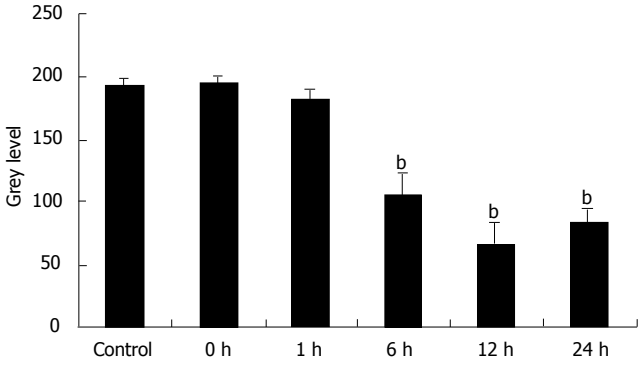


Figure 5 The synthesis of MIP-1α protein in Kupffer cells tested by immunohistochemical (n = 8, mean ± SE, $^bP < 0.01$ compared with control group).

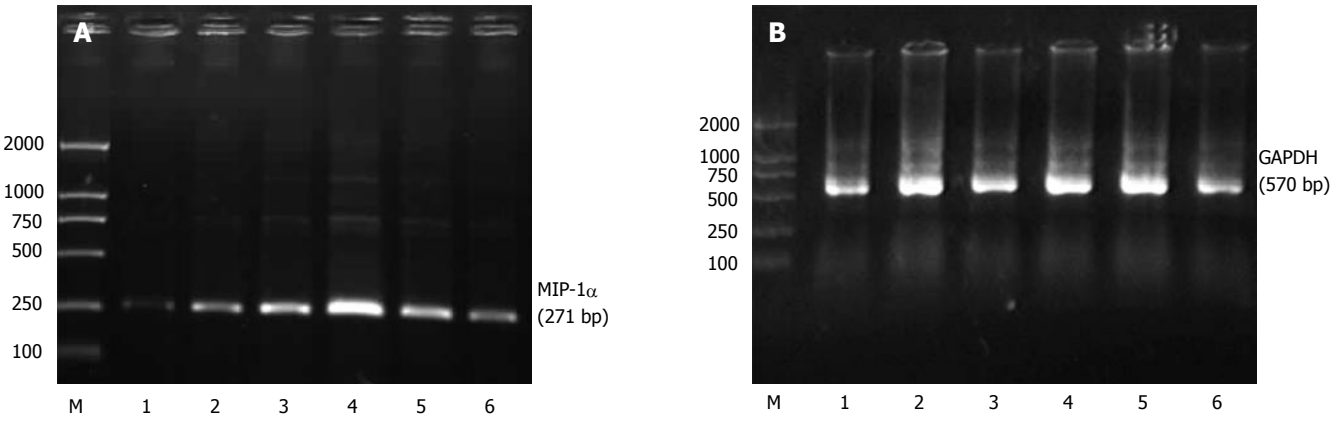


Figure 6 **A:** Photograph of the MIP-1α mRNA expression in Kupffer cells tested by RT-PCR; **B:** Photograph of the GAPDH mRNA expression in Kupffer cells tested by RT-PCR.

modles were used to establish the pathological process of hepatic ischemia/reperfusion injury, such as the liver transplantation model, the partial warm or cold ischemia/reperfusion injury model and the total hepatic ischemia/reperfusion injury model[16-18]. In this study, the model of rat liver ischemia-reperfusion injury is established by the clamping and unclamping of the vessels to the left lateral and median hepatic lobes, which account for 70% of the rat's liver mass. This hepatic insult is similar to the clinical situation where the whole liver is rendered ischemic during total vascular exclusion (which is not tolerated in the rat) for liver resections. We determine the outcomes 0 h, 1 h, 6 h, 12 h and 24 h respectively after the onset of reperfusion, when a number of mechanisms began to function, such as the increase in the cytokine production, the enhanced oxidative metabolism, and the increase in phagocytotic activity, which contribute to the hepatic injury[1,2]. Therefore, the findings of the present research reveal the pathological process of both 'early and late' phase ischemia/reperfusion injury.

Kupffer cells play an important part in mediating ischemia and reperfusion injury. When activated during the ischemia and subsequent reperfusion, they generate excessive inflammatory cytokines and oxygen-derived free radicals, which play a particularly important role in the pathogenesis of hepatic ischemia and reperfusion injury[19]. In this study, low levels of TNF-α and IL-1β were detected in the supernatant of primary cultures of rat Kupffer cells

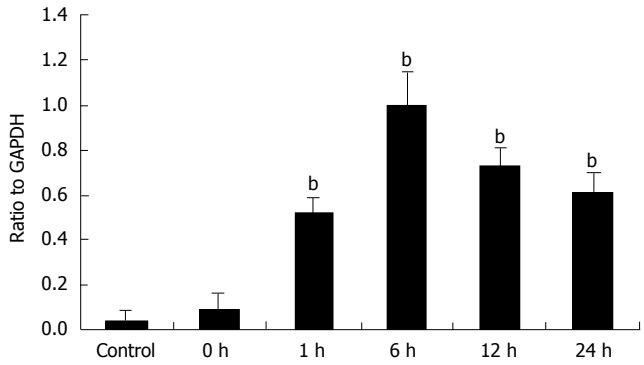


Figure 7 Quantitative data of MIP-1α mRNA levels (n = 8, mean ± SE, $^bP < 0.01$ compared with control group).

in the control group. At the early phase of the ischemia/reperfusion injury, they significantly increased and nearly reached its maximum. Twenty-four h after the reperfusion, these inflammatory cytokines were still significantly high compared with that in the control group. The mechanism of induction of TNF-α and IL-1β bioactivity after hepatic ischemia/reperfusion is complex. As a survival factor in hepatocytes under certain physiological conditions, for example, liver regeneration, Kupffer cells secrete low levels of TNF-α and IL-1β. During the very early phase of the

hepatic ischemia/reperfusion injury, the activated Kupffer cells produce high concentrations of TNF-α and IL-1β that represents the results obtained 0 h and 1 h after the reperfusion. The secretions of these toxic inflammatory cytokines precedes the activation of adhesion factors, chemotactic agents, and the sequestration of neutrophils in the liver and appear to be a key mediator of the inflammatory response to promote the release of other cytokines.

Kupffer cells are both a target and a source of chemokines. Therefore, we also examine the expression of MIP-1α, an important member of CC chemokine subfamily, in Kupffer cells in an ischemia/reperfusion injury. The MIP-1α proteins are not detected by immunocytochemiscal staining in the cells of the control group and IRI group I and II. Likewise, the synthesis of MIP-1α mRNA in the cells of the control group and IRI group I are not active. After the reperfusion injury, the expression of MIP-1α protein and mRNA significantly increase. This may indicate that the genes MIP-1α are inactive in normal Kupffer cells. Some inflammatory factors stimulate MIP-1α gene in Kupffer cells during the reperfusion injury. These findings are in agreement with those in the research by Bukara M *et al*[20]. The role of MIP-1α in the pathological process of hepatic ischemia/reperfusion injury is not well known. However, previous studies have proved that MIP-1α can induce mononuclear macrophage to secrete TNF-α and IL-1β[11]. Therefore, we suppose the active behavior of the MIP-1α gene in the Kupffer cell following liver ischemia/reperfusion injury is assumed to be one of the major causes for the hepatic ischemia/reperfusion injury.

In conclusion, our study focuses on the immediate, innate immune response of the Kupffer cells and demonstrates the prominent expression of MIP-1α in the Kupffer cells in the hepatic ischemia/reperfusion injury. These theories may provide a better understanding of the mechanisms of hepatic ischemia/reperfusion injury and will be useful for the therapies of hepatic ischemia/reperfusion injury in the fields of hepatic surgery, preservation, and rejection.

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BASIC RESEARCH

High efficient mammalian expression and secretion of a functional humanized single-chain Fv/human interleukin-2 molecules

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Abstract

AIM: To construct and produce a recombinant bispecific humanized single-chain Fv (sFv) /Interleukin-2 (IL-2) fusion protein by using mammalian cells.

METHODS: The sFv/IL-2 protein was genetically engineered, and transfected to mammalian cells to determine whether the mammalian protein folding machinery can produce and secrete active sFv/IL-2 with high efficiency.

RESULTS: The fusion protein was constructed and high efficiently expressed with yields up to 102 ± 4.2 mg/L in culture supernatant of the stably transfected 293 cell line. This recombinant fusion protein consisted of humanized variable heavy (V_H) and light (V_L) domains of monoclonal antibody (mAb) 520C9 directed against the human HER-2/neu (c-erbB2) proto-oncogene product p185, and human IL-2 connected by polypeptide linker. The fusion protein was shown to retain the immunostimulatory activities of IL-2 as measured by IL-2-dependent cell proliferation and cytotoxicity assays. In addition to its IL-2 activities, this fusion protein also possessed antigen-binding specificity against p185, as determined by indirect ELISA using p185 positive SKOV 3ip1 cells.

CONCLUSION: The large-scale preparation of the recombinant humanized sFv antibody/IL-2 fusion protein is performed with 293 cells. The recombinant humanized sFv antibody/IL-2 fusion protein may provide an effective means of targeting therapeutic doses of IL-2 to p185 positive tumors without increasing systemic toxicity or immunogenicity.

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Key words: Interleukin-2; Humanization; Antibody; Fusion protein; HER-2/neu

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INTRODUCTION

Interleukin-2 (IL-2) is a 15 kD cytokine synthesized and secreted primarily by activated T cells[1,2]. IL-2 plays a central role in the augmentation of cell-mediated immune responses by stimulating and supporting a broad range of immune cells, including T and B cells, macrophages, and natural killer (NK) cells[3]. Furthermore, IL-2 can also stimulate the production of lymphokine-activated killer (LAK) cells, which are derived from NK cells and can effectively mediate antibody-dependent cellular toxicity. The administration of recombinant human IL-2 (rhIL-2), in combination with LAK cells, has been shown to be of value in the treatment of patients with advanced melanoma or renal cell carcinoma[4]. However, the *in vivo* efficacy of rhIL-2 treatment has been limited by its associated severe systemic toxicity and difficulties in maintaining prolonged high concentration of the cytokine in the tumor microenvironment, which is likely necessary to induce local anti-tumor immune responses[5,6]. To circumvent these problems, several approaches to selectively target IL-2 to tumor sites have been employed, particularly the use of immunoconjugates[7-9].

Murine monoclonal antibodies (mAbs) have been extensively used as carriers to target therapeutic agents to tumor sites for diagnostic and therapeutic modalities over the past decade. Despite some highly encouraging diagnostic data obtained with this approach, the general therapeutic efficacy has been rather disappointing[10-13]. Several major obstacles related to the mAb approach

have been identified, including relatively long half-life of the immunocomplex, human anti-mouse antibody (HAMA) response and inability of the immunoconjugate to penetrate large tumor masses[14,15]. To date, several approaches of engineering conventional murine mAbs have been developed for more effective cancer targeting therapy. One approach is the development of Fv portion of an antibody consisting of the VH and VL domains. This version of antibody is the smallest antibody fragment to bear the antigen binding site. Furthermore, the reports showed that a genetically engineered single-chain Fv(sFv) with binding activity could be produced by connecting the carboxyl terminus of one V domain to the amino terminus of the other with a flexible peptide linker[16]. Advantages of this small antibody fragment include improved clearance of immunocomplex from the circulation, better penetration in solid tumors and lower immunogenicity[17-20]. Other approaches include the development of recombinant immunotoxins and antibody-immunostimulatory molecule conjugates[21-24].

In this study we constructed genetically a recombinant bispecific fusion protein, sFv/IL-2 consisting of sFv and IL-2 portions. We hoped that this protein could target IL-2 to tumor sites to overcome those obstacles mentioned above. The kind of recombinant proteins could be generated in bacteria. But protein from bacteria is not active and must be solubilized, oxidized, and renatured *in vitro*. Even so it is impossible to avoid the loss of its bioactivities. A different approach to produce active fusion protein would be to use the more sophisticated refolding machinery that is located in the endoplasmic reticulum (ER) of mammalian cells. But, fusion proteins were usually expressed at very low level in mammalian cells thus it was not efficient for large-scale preparation[25-27]. For these reasons, we tried to find out an efficient mammalian expression system for the large-scale preparation of the recombinant humanized sFv antibody/hIL- 2 fusion protein[28], and an effective method for preparing the therapeutic doses of the fusion protein.

MATERIALS AND METHODS

Construction of expression plasmids

Mammalian expression plasmids encoding the humanized sFv antibody/IL-2 fusion proteins were constructed. Baculovirus expression plasmid pAcHCs-520C9sFv containing a humanized 816-bp 520C9 sFv cDNA fragment, and the plasmids pLW46 and plasmid pLW42 containing either wild type or mutant human IL-2 cDNA fragments, respectively, were kindly provided by Chiron Corporation (Emeryville, CA). The mutant human IL-2 cDNA contains a single base substitution at position 173 (G→C) which converts amino acid Cys 58 of the wild type protein to Ser, thus eliminating a disulphide bond critical to the biological function of IL-2. Primers used for the amplification of 520C9 sFv and human IL-2 cDNA fragments from the above plasmids were synthesized at the Institute for Molecular Biology (MOBIX), McMaster University (Hamilton, ON). The forward primer AB9883 (5'-CTT AAG CTT GCC ACC ATG GAC ATG AGG GTC CCC GCT-3') was used to amplify the sFv cDNA containing a Kozak consensus sequence prior to the initiating ATG and a terminal *Hin*dIII restriction site. The reverse primer AB7824 (5'-CC GAA TTC TTT AAT CTC CAG TTT TGT CCC TTG GGC-3') contained an *Eco*RI site 3' to the sFv cDNA fragment. The 440-bp mutant human IL-2 and wild type human IL-2 cDNA fragments derived from pLW42 and pLW46 were subjected to PCR amplification using the forward primer AB7822 (5' -GGG GAA TTC GGT GGC GGT GGC TCG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT ATG CCT ACT TCA AGT TCT ACA AAG AAA ACA CAG-3'), which contained a terminal *Eco*RI site and a coding sequence for a 15 amino acid, glycine rich linker peptide (NH_2-GGGGSGGGGSGGGGS-COOH) 5' to the human IL-2 cDNA fragment. The reverse primer AB4749 (5'-GAC CTC GAG TCA GTG TTG AGA TGA T-3') contained a terminal *Xho*I site adjacent to the termination codon of the human IL-2 cDNA fragment. PCR products were purified from 0.8% agarose gels and ligated into T-ended pBluescript using T4 DNA ligase (Gibco/BRL, Burlington, ON). The ligation mixture was then used to transform competent DH5α cells (Gibco/BRL). Plasmids with inserts were digested with *Hin*dIII and *Eco*RI for the sFv, or *Eco*RI and *Xho*I for the human IL-2 cDNA fragments. These cDNA fragments were purified from agarose gels using QIAGEN Gel purification kit (Qiagen, Chatsworth, CA) and ligated into the *Hin*dIII/*Xho*I site of the mammalian expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA) to produce the recombinant plasmids pcDNA-H520C9sFv-hIL-2 and pcDNA-H520C9sFv-mhIL-2. Fidelity of the PCR-amplified constructs was confirmed by fluorescence-based double-stranded DNA sequencing (MOBIX). The ligation mixture was then used to transform competent DH5α cells. Transformants containing both the humanized 520C9 sFv and IL-2 cDNA fragments were confirmed by restriction enzyme analysis. The constructs were subsequently purified using QIAGEN plasmid Midi kits and dissolved in Tris-EDTA buffer (pH 7.4) to a concentration of 0.4 mg/mL.

Establishment of stable cell lines

The 293 cells used for stable expression of the cDNAs encoding H520C9sFv-hIL-2 or H520C9sFv-mhIL-2 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL) containing 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT) at 37℃ in a 50 mL/L CO_2 incubator. Cells grown to 20% confluency were transfected with 5 μg of the plasmid constructs using 30 μL of SuperFect transfection reagent (Qiagen) as described by the manufacturer. As a negative control, pcDNA3.1 (+) was used to transfect 293 cells under the same conditions. Stable transfectants were selected in DMEM containing 10% FCS and 600 μg/mL G418 (Gibco/BRL) for two weeks, after which the concentration of G418 was increased to 1.2 mg/mL. Clones secreting the H520C9sFv-hIL-2 fusion protein were identified by ELISA using either a mouse anti-human IL-2 mAb MAB202 (R&D Systems, Minneapolis, MN) or a rabbit anti-human IL-2 polyclonal antibody EP100 (Genzyme, Cambridge, MA). In brief, 100 μL of supernatant from different clones was

added to a 96-well plate coated with MAB202 (1:250 dilution), and incubated at 37℃ for 2 h. After three washes with PBST, the plate was incubated with EP100 (1:200 dilution) at 37℃ for 1 h, washed and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (Gibco/BRL) (1:1000 dilution) for 1 h at 37℃. After three additional washes with PBST, colour development was performed with peroxidase substrate (Bio-Rad, Hercules, CA) and the resulting OD determined at 405 nm in a microplate reader (Bio-Tek Instrument Inc., Winooski, VT). Clones producing the highest amount of fusion protein were selected and grown in DMEM containing 10% FCS for 72 h. The conditioned medium was collected for subsequent precipitation and analysis.

Determination of concentration of fusion protein

The quantitative indirect ELISA was performed to determine the concentration of fusion protein of conditioned medium. A 96-well microtiter plate was coated with MAB202 (1:250 dilution), and incubated at 37℃ for 2 h overnight at 4℃. The 100 μL of serial diluted standard rhIL-2 and diluted conditioned medium were then added to each well, and incubated at room temperature for 2 h. After three washes with PBST, the plate was incubated with EP100 (1:200 dilution) at 37℃ for 1 h, washed and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (Gibco/BRL) (1:1000 dilution) for 1 h at 37℃. After three additional washes with PBST, colour development was performed with peroxidase substrate and the resulting OD determined at 405 nm in a microplate reader as above.

Immunoprecipitation and purification of fusion proteins

H520C9sFv-hIL-2 and H520C9sFv-mhIL-2 fusion proteins were immunoprecipitated using MAB202 coupled with CNBr-activated Sepharose CL-4B (Pharmacia Biotech, Baie d'Urf, QC). Twenty-five microliters of a 1:1 (v/v) slurry of the absorbent suspended in 20 mmol/L Tris-HCl buffer (pH 7.8) were added to 1.5 mL of the conditioned medium with gentle shaking at 4℃ for 4 h. The Sepharose beads were washed three times using Tris-HCl buffer, then 50 μL of 1 × SDS-PAGE sample buffer containing β-mercaptoethanol was added to the beads. After heating at 100℃ for 3 min, the beads were pelleted by centrifugation and the supernatant collected for immunoblot analysis.

For affinity purification of the fusion protein, the conditioned medium was applied to an MAB202-Sepharose CL-4B affinity column at a flow rate of 5 column volume per h. The column was then washed three times with 20 mmol/L Tris-HCl buffer (pH 7.8) and the fusion protein was eluted with Pierce Gentle Ag/Ab Elution Buffer (Rockford, IL) as described by the manufacturer.

Immunoblot analysis

Twenty microliters of the above supernants were separated on a 15% SDS-polyacrylamide gel under reducing conditions. Proteins were electroblotted onto nitrocellulose membranes (Bio-Rad, Richmond, CA) and the efficiency of protein transfer was determined by staining the blots with Ponceau S (Sigma, St. Louis, MO). Membranes were washed briefly in TBST to remove the Ponceau S stain, blocked in TBST containing 5% skimmed milk at 4℃ overnight and incubated at room temperature for 2 h with EP100 (1:1000 dilution). Following three 20 min washes in TBST, the membranes were incubated for 2 h with an HRP-conjugated goat anti-rabbit IgG polyclonal antibody (1:2000 dilution). After washing in TBST, membranes were developed with the Renaissance Chemiluminescence Reagent kit (DuPont, Boston, MA) as described by the manufacturer.

IL-2 bioactivity assay

Biological activity of the fusion proteins was determined by standard IL-2-dependent CTLL-2 cell proliferation[2] and cytotoxicity assays[29]. CTLL-2 cells deprived of rhIL-2 (Chiron, Emeryville, CA) for 3 d were seeded into a 96-well plate at 1×10^4 cells per well and incubated with either serially diluted rhIL-2 standard or fusion protein at 37℃ in a 50 mL/L CO_2 incubator. After 24 h, 1 μCi of [^{3}H]-thymidine (DuPont, Boston, MA) was added to each well and the cells incubated for an additional 12 h. Cells were harvested onto glass fibre filters (Whittaker Corp., Walkersville, MD), dried and the incorporated radioactivity was counted in a Beckman scintillation counter (Fullerton, CA).

For bioactivity neutralization experiments, 2 ng/mL of rhIL-2 and the same concentration of IL-2 in H520C9sFv-hIL-2 were incubated with increasing concentrations of the anti-rhIL-2 neutralizing mAb MAB202 for 2 h at 37℃ in a 96-well microtiter plate. As a control, mouse IgG was added to H520C9sFv-hIL-2 instead of MAB202. Following this incubation period, 1×10^4 CTLL-2 cells were added to each well. The assay mixture, in a total volume of 100 μL/well, was incubated for 24 h at 37℃ in a 50 mL/L incubator and pulsed with 1 μCi/well [^{3}H]-thymidine for the final 12 h. The cells were then harvested onto glass fiber filters and [^{3}H]-thymidine incorporation was determined.

In the cytotoxicity assay, peripheral blood mononuclear cells (PBMCs) from healthy individuals were separated on a Histopaque 1077 density gradient (Sigma, St. Louis, MO). The PBMCs were cultured in RPMI 1640 medium (Gibco/BRL) supplemented with 10% FCS containing either 1000 IU/mL rhIL-2 or same dose of the H520sFv-hIL-2 fusion protein as determined by the above CTLL-2 assay for 3 d to produce LAK cells. Cytotoxicity of the resulting LAK cells was assessed by a 4 h Calcein AM (Molecular Probe, Eugene, OR) release assay with NK-resistant Daudi cells (ATCC, Rockville, MD) used as target cells. Each well of a 96-well plate contained 1×10^4 Daudi cells which had been pre-labelled with fluorescent Calcein AM. To achieve different effector/target cell ratios, an increasing number of LAK cells was added to each well. The total volume of culture medium per well was 0.1 mL. Following co-incubation of LAK and Daudi cells at 37℃ for 4 h, the cells were pelleted and the fluorescent intensity value ($Value_{sample}$) of 50 μL of cell supernatant from each well containing both types of cells was measured with a Cytofluor 2300 fluorescence reader system (Millipore,

Bedford, MA). The maximal release value ($Value_{maximal}$) of the fluorescent tag was determined by adding an equal volume of 1% (v/v) Triton X-100 to the wells with only target cells. The spontaneous release value ($Value_{spontaneous}$) of the tag was given by the fluorescent intensity of 50 μL of cell supernatant from the wells without any LAK cells. The percentage of specific lysis for each sample of LAK cells was calculated by the formula ($Value_{sample}$-$Value_{spontaneous}$)/($Value_{maximal}$-$Value_{spontaneous}$) × 100.

Determination and specificity of antigen-binding activity of H520C9sFv-hIL-2

Antigen-binding activity was measured by indirect ELISA using cultured SKOV3ip1. Human ovarian carcinoma SKOV3ip1 cells which express high levels of p185[30] were maintained in DMEM containing 10% FCS and used for determination of antigen-binding activity of the H520C9s-Fv-hIL-2 fusion protein. Cells (1×10^4) were added to individual wells of a 96-well microtiter plate one day before the assay. Serially diluted samples of the H520C9sFv-hIL-2 or a chemically-conjugated control mAb 520C9-rhIL-2 were added to each well of the plate and incubated at 37℃ for 2 h[31]. After three washes with PBST, EP100 (1:250 dilution) was added to the cells and incubated for 2 h. Following three washes with PBST, HRP-conjugated goat anti-rabbit IgG polyclonal antibodies (Gibco/BRL) (1:1000 dilution) were added to the cells. After 2 h incubation, the cells were washed three times with PBST, colour was developed with the addition of peroxidase substrate (Bio-Rad, Hercules, CA) and the OD of the solution was determined at 405 nm in a microplate reader as described.

To demonstrate specificity for p185 binding, SKOV 3ip1 cells (1×10^4) grown in 96-well plates were treated with either serially diluted intact 520C9 mAb or normal mouse IgG (0.001 to 10 nmol/L) at 37℃ for 2 h prior to the addition of 10 nmol/L H520C9sFv-hIL-2 fusion protein. Indirect ELISA was then performed as described above.

Statistical analysis

Data are presented as mean ± SD. Statistical differences between groups were determined by using Student's *t*-test. For all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Determination of concentration of the fusion protein

The concentration of conditioned media from 293 cells stably transfected with either pcDNA-H520C9sFv-hIL-2 or pcDNA-H520C9sFv-mhIL-2 were at 102.0 ± 4.2 or 101.0 ± 5.6 mg/L, respectively.

Detection of the IL-2 moiety in the fusion protein

To confirm the presence of the IL-2 moiety in the fusion protein, the MAB202 immunoprecipitates of conditioned media from 293 cells stably transfected with either pcDNA3.1(+), pcDNA-H520C9sFv-hIL-2 or pcDNA-H520C9sFv-mhIL-2 were analysed by Western blotting using EP100. As shown in Figure 1, a single band of

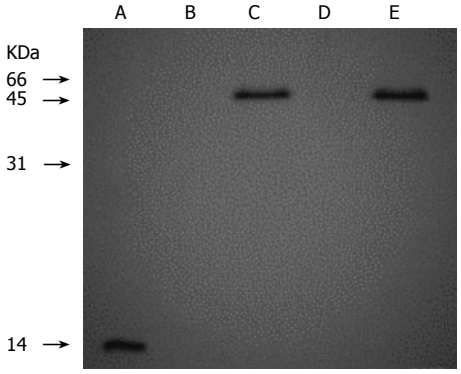


Figure 1 Western blot analysis of recombinant humanized sFv antibody/IL-2 fusion proteins stably expressed in 293 cells. Lane A: 0.5 μg of rhIL 2; Lanes B, C, D and E: The supernants from conditioned medium of 293 cells, 293 cells transfected with pcDNA-H520C9sFv-hIL-2, pcDNA3.1 (+) and pcDNA-H520C9sFv-mhIL-2, respectively. Samples were separated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to nitrocellulose membranes and immunoblotted using the anti-human IL-2 polyclonal antibody, EP100. The fusion proteins, H520C9sFv-hIL-2 and H520C9sFv-mhIL-2 were shown to migrate as single bands of 45 kD.

45 kD was observed in the conditioned media from 293 cells transfected with either pcDNA-H520C9sFv-hIL-2 (lane C), or pcDNA-H520C9sFv-mhIL-2 (lane E), but not in the culture supernatant of 293 cells (lane B) or the conditioned medium from 293 cells transfected with pcDNA3.1(+) (lane D). Furthermore, the migration positions of these fusion proteins on SDS-PAGE were consistent with their predicted molecular mass. As a positive control, EP100 also recognized rhIL-2 (lane A).

Cell proliferation and cytotoxicity assays

The activity of the H520C9sFv-hIL-2 fusion protein to support the proliferation of IL-2-dependent CTLL-2 cells and to generate LAK cells was compared to those of rhIL-2. As shown in Figure 2, conditioned medium from 293 cells transfected with pcDNA-H520C9sFv-hIL-2, but not pcDNA-H520C9sFv-mhIL2, possessed similar IL-2 bioactivity to rhIL-2 standard. These results indicated that the bioactivity of the IL-2 moiety in terms of its ability to support the growth of CTLL-2 cells was maintained in the H520C9sFv-hIL-2 fusion protein.

In the cytotoxicity assay directed against target Daudi cells, LAK cells generated by 1000 IU/mL of IL-2 of the H520C9sFv-hIL-2 fusion protein, as determined by the above CTLL-2 assay, demonstrated similar effect in the killing of Daudi cells compared to LAK cells activated by 1000 IU/ml of rhIL-2 (Figure 3). The specific lyses of Daudi cells by LAK cells stimulated by the H520C9sFv-hIL-2 and rhIL-2 were 79% and 77%, respectively, at an effector : target ratio of 125:1. No cytotoxic effect was observed when conditioned medium from control 293 cells transfected with pcDNA3.1(+) was used.

Neutralization of IL-2 activity in H520C9sFv-hIL-2

To determine if the proliferation effects of the H520C9sFv-hIL-2 fusion protein were strictly IL-2-dependent, inhibition studies using IL-2 neutralizing antibodies were performed. As shown in Figure 4A, the

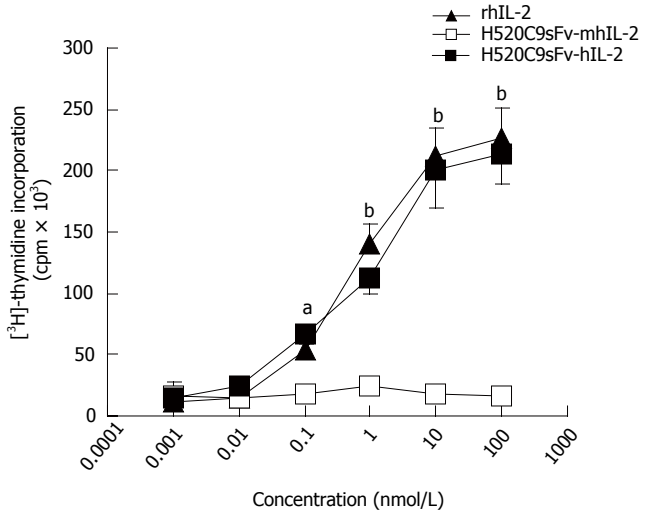


Figure 2 IL 2 activity of recombinant humanized sFv antibody/IL-2 fusion proteins. IL 2 activity of rhIL-2, or conditioned medium from 293 cells transfected with either pcDNA-H520C9sFv hIL 2 or pcDNA-H520C9sFv mhIL 2 was measured by [^{3}H] thymidine incorporation in CTLL 2 cells. The data are the mean ± SD from three separate experiments. $^bP < 0.01$ for rhIL-2 *vs* H520C9sFv mhIL 2, and $P > 0.05$ *vs* H520C9sFv hIL 2; $^aP < 0.05$ for rhIL-2 *vs* H520C9sFv mhIL 2, and $P > 0.05$ *vs* H520C9sFv hIL 2.

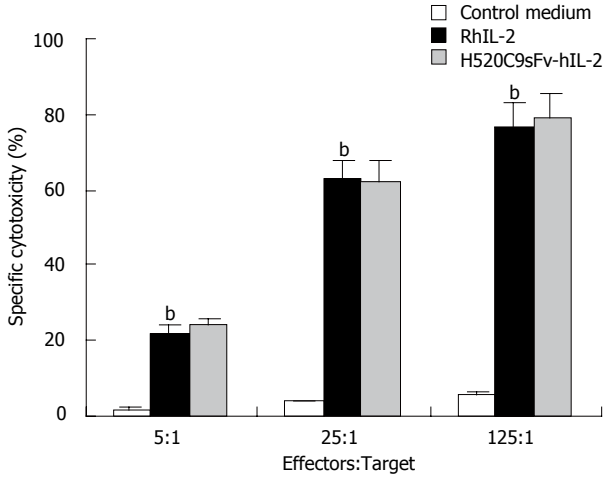


Figure 3 LAK cell-mediated cytotoxicity induced by H520C9sFv-hIL-2 fusion protein. Human LAK cells (effector) were generated by incubation of fresh PBMCs with either conditioned medium from control 293 cells transfected with pcDNA 3.1 (+), rhIL 2 or equivalent dose of H520C9sFv hIL 2. Lysis of Daudi cells (target) was determined using a 4 h Calcein AM release assay. The data are the mean ± SD from three separate experiments. $^bP < 0.01$ for rhIL-2 *vs* control medium, and $P > 0.05$ *vs* H520C9sFv hIL 2.

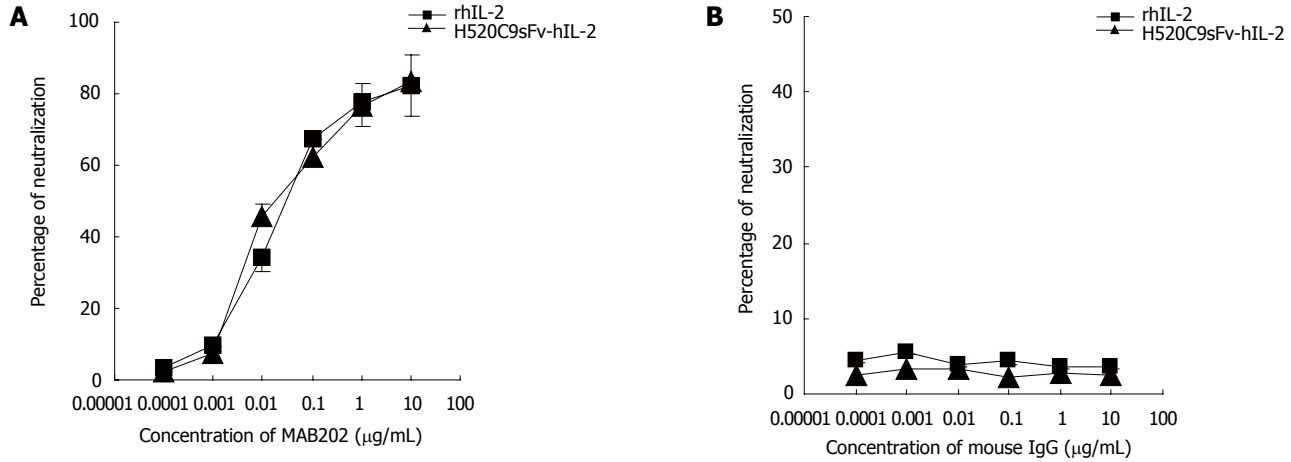


Figure 4 Inhibition of stimulatory effects of rhIL 2 or H520C9sFv hIL 2 on CTLL 2 cells by the anti human IL 2 neutralizing antibody, MAB202. Based on the molecular mass of either IL-2 or H520C9sFv-hIL-2 fusion protein, the neutralization dose 50 for 2 ng/ml rhIL 2 or the same dose of H520C9sFv hIL 2 was determined to be approximately 0.0176 and 0.022 μg/mL MAB202, respectively (**A**). No inhibitory effect was observed using control mouse IgG (**B**). The data are the mean ± SD from three separate experiments (**B**). **A**: $P > 0.05$ for rhIL-2 *vs* H520C9sFv hIL 2; **B**: $P > 0.05$ for rhIL-2 *vs* H520C9sFv hIL 2.

proliferative effects of both rhIL-2 and H520C9sFv-hIL-2 on CTLL-2 cells, as measured by [^{3}H]-thymidine incorporation of the CTLL-2 cells were inhibited by MAB202. Approximately 50% inhibition of [^{3}H]-thymidine incorporation for 2 ng/mL rhIL-2 or same dose of H520C9sFv-hIL-2 was achieved at an MAB202 concentration of approximately 0.0176 and 0.022 μg/mL, respectively. No inhibitory effect was observed when control mouse IgG was added (Figure 4B).

Antigen-binding assays

The antigen-binding specificity of the H520C9sFv-hIL-2 fusion protein was determined by indirect ELISA using SKOV 3ip1 cells, which overexpressed p185. As shown in Figure 5, the conditioned medium from 293 cells transfected with either pcDNA-H520C9sFv-hIL-2 or pcDNA-H520C9sFv-mhIL-2 displayed binding activity for p185 on the cell surface of SKOV 3ip1 cells. As a positive control, the parental 520C9 mAb chemically conjugated to rhIL-2 was shown to bind SKOV 3ip1 cells.

To confirm that the binding of H520C9sFv-hIL-2 fusion protein was specific for p185, SKOV 3ip1 cells were exposed to either the parental 520C9 mAb or normal mouse IgG prior to indirect ELISA. As shown in Figure 6, 520C9 mAb was able to inhibit the binding of the fusion protein to cells in a dose-dependent manner. In contrast, normal mouse IgG did not inhibit the binding of the fusion protein.

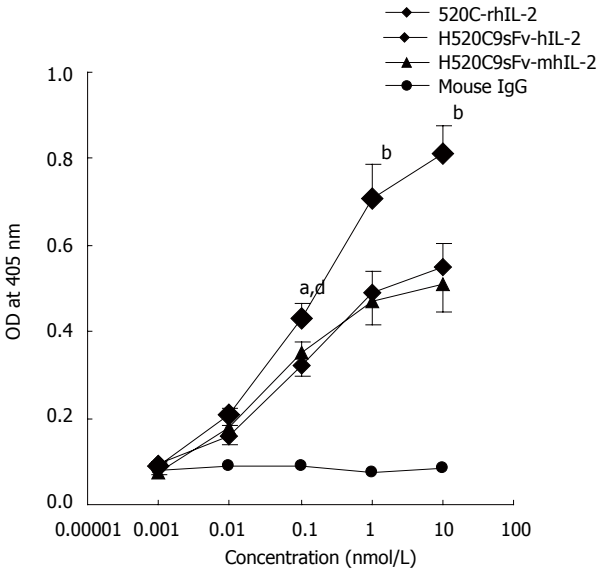


Figure 5 Determination of antigen-binding activity of H520C9sFv-hIL-2 fusion protein. Antigen binding activity was measured by indirect ELISA using cultured SKOV 3ip1. Bound fusion protein was recognised with the anti-human IL2 polyclonal antibody EP100. The fusion proteins, H520C9sFv-hIL-2 and H520C9sFv-mhIL-2 were shown to bind to p185 positive cells SKOV 3ip1. A chemically-conjugated molecule containing the intact parental 520C9 mAb and rhIL-2(520C9-rhIL-2) was used as a positive control. As a negative control, mouse IgG failed to show any binding activity. The data are the mean ± SD from three separate experiments. $^{b}P < 0.01$ *vs* mouse IgG; $^{b}P < 0.01$ for 520C9-rhIL-2 *vs* H520C9sFv-hIL-2 and H520C9sFv-mhIL-2; $^{d}P < 0.01$ *vs* mouse IgG; $^{a}P < 0.05$ for 520C9-rhIL-2 *vs* H520C9sFv-hIL-2 and H520C9sFv-mhIL-2.

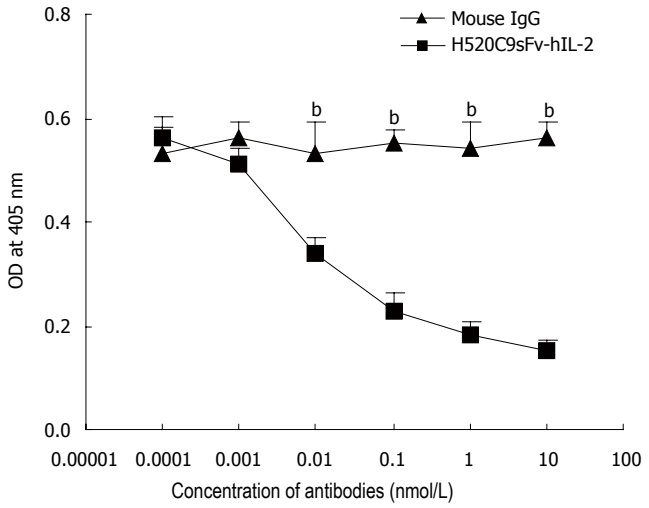


Figure 6 Anti-p185 mAb, 520C9, specifically blocks the binding of H520C9sFv hIL 2 fusion protein to SKOV 3ip1 cells. SKOV 3ip1 cells, pre-exposed to serially diluted intact 520C9 monoclonal antibody or to mouse IgG (0.0001 to 10 nmol/L), then incubated with 10 nmol/L H520C9sFv hIL 2 fusion protein. Bound fusion protein was measured by indirect ELISA using the anti human IL 2 polyclonal antibody, EP100. 520C9 mAb caused a dose-dependent decrease in the binding of the fusion protein to SKOV 3ip1 cells. Control mouse IgG had no effect on the binding of the fusion protein. The data are the mean ± SD from three separate experiments. $^{b}P < 0.01$ *vs* mouse IgG, $P > 0.05$ *vs* mouse IgG.

DISCUSSION

In this study, we report the construction, high efficient expression and characterisation of a recombinant humanized sFv antibody/hIL-2 fusion protein, H520C9sFv-hIL-2. The humanized sFv of 520C9 was constructed by introducing into a human sFv the amino acids from the complementarity-determining regions of mouse mAb 520C9, that recognize the HER/neu (c-erbB2) proto-oncogene product, p185[32]. Human IL-2 was then fused to the C-terminus of H520C9sFv by a flexible glycine linker. Following stable expression in 293 cells, this fusion protein was shown to retain both the immuno-stimulatory activities of IL-2 and the p185 binding characteristics of the parental 520C9 mAb. In the supernatant from stably transfected 293, the concentration of fusion protein cells was demonstrated to be at 102.0 ± 4.2 mg/L. This milligram scale expression makes it possible to prepare a large-scale fusion protein from mammalian cells, which is usually beneficial for keeping the bioactivities of fusion protein.

Our experiments demonstrated no loss of activity of IL-2 in the H520C9sFv-hIL-2 fusion protein as measured by IL-2-dependent cell proliferation and cytotoxicity assays, compared to rhIL-2 standard. This suggests that the fusion of IL-2 to the C-terminus of the humanized 520C9 sFv does not affect the biological activity of IL-2 and is consistent with previous studies describing the preparation of Fab'-IL-2 and SCA-IL-2 fusion proteins[33,34]. Retention of the IL-2 activity in H520C9sFv-hIL-2 may be partially due to the flexible glycine-rich linker between the C-terminus of sFv and the N-terminus of IL-2 which allows for proper folding of the IL-2 moiety, thereby enabling it to interact with its receptor. This is supported by previous findings with diphtheria toxin-IL-2 fusion proteins demonstrating the importance of the mobility of IL-2 in allowing its interaction to occur effectively[35].

We have developed a humanized sFv antibody/human IL-2 fusion protein, which was generated in mammalian cells with high efficacy and shown to retain both IL-2 activity and p185 binding affinity. The use of a humanized sFv as the fusion partner over other antibody-based molecules has the potential to reduce the immunogenicity of the fusion protein. In addition, the small size of the sFv fragment could likely improve its pharmacokinetic properties with respect to penetration of solid tumors and clearance. This bifunctional H520C9sFv-hIL-2 fusion protein may thus provide an effective method of targeting therapeutic doses of IL-2 to tumors or other targeted cells with significantly decreased side-effects of IL-2 and its immunogenicity.

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CLINICAL RESEARCH

Efficacy of balloon-occluded retrograde transvenous obliteration, percutaneous transhepatic obliteration and combined techniques for the management of gastric fundal varices

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Abstract

AIM: To evaluate the effect of three interventional treatments involving transvenous obliteration for the treatment of gastric varices, and to compare the efficacy and adverse effects of these methods.

METHODS: From 1995 to 2004, 93 patients with gastric fundal varices underwent interventional radiologic embolotherapy at our hospital. Of the 93 patients, 75 were treated with the balloon-occluded retrograde transvenous obliteration (BRTO) procedure; 8 were with the percutaneous transhepatic obliteration (PTO) procedure; and 10 were with the combined BRTO and PTO therapy. A follow-up evaluation examined the rates of survival, recurrence and rebleeding of the gastric varices, worsening of esophageal varices and complications in each group.

RESULTS: The BRTO, PTO, and combined therapy were technically successful in 81% (75/93), 44% (8/18), and 100% (10/10) patients, respectively. Recurrence of gastric varices was found in 3 patients in the BRTO group and in 3 patients in the PTO group. Rebleeding was observed in 1 patient in the BRTO group and in 1 patient in the PTO group. The 1- and 3-year survival rates were 98% and 87% in the patients without hepatocellular carcinoma (HCC) in the BRTO group, 100% and 100% in the PTO group, and 90% and 75% in the combined therapy group, respectively.

CONCLUSION: Combined BRTO and PTO therapy may rescue cases with uncontrollable gastric fundal varices that remained even after treatment with BRTO and/or PTO, though there were limitations of our study, including retrospective nature and discrepancy in sample size between the BRTO, PTO and combined therapy groups.

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Key words: Gastric varices; Balloon-occluded retrograde transvenous obliteration; Percutaneous transhepatic obliteration; Combined therapy

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INTRODUCTION

Currently, various types of therapeutic procedures, including surgery, endoscopic procedures, and direct therapeutic intervention, are available for the treatment of gastric varices. Interventional therapy for gastric varices is classified as transjugular intrahepatic portosystemic shunt (TIPS) and transcatheter embolotherapy. Many studies have reported the use of TIPS in patients with gastric varices[1-5]. Although interventional embolotherapy has been applied for the treatment of gastric varices and a recent study has shown that embolotherapy may control gastric varices better than TIPS, treatment indications and a recommended embolotherapeutic strategy for the eradication of gastric fundal varices have not yet been strictly established[5-10]. In this study, we evaluated the effect of three interventional treatments involving transvenous obliteration for the treatment of gastric varices; the efficacy and adverse effects of these methods were compared. The present study involved 93 patients in whom interventional embolotherapy was performed as a treatment for gastric fundal varices. In addition, 10 of the 93 patients were treated with a combined balloon-occluded retrograde

Table 1 Main clinical and biochemical characteristics of 93 patients treated with interventional radiology for gastric varices *n* (%)

Clinical characteristics	BRTO	PTO	Com-T	Total
Sex (M/F)	37/38	5/3	5/5	47/46
Age (mean ± SD)	63.4 ± 10.0	62.9 ± 8.4	68.9 ± 12.8	63.9 ± 10.2
Cause of portal hypertension				
Alcoholic liver cirrhosis	10 (13)	1 (13)	3 (30)	14 (15)
HBV (+) liver cirrhosis	3 (4)	0 (0)	1 (10)	4 (4)
HCV (+) liver cirrhosis	52 (69)	6 (75)	5 (50)	63 (68)
HBV (-) HCV (-) liver cirrhosis	7 (9)	0 (0)	1 (10)	8 (9)
Idiopathic portal hypertension	1 (1)	1 (13)	0 (0)	2 (2)
Primary biliary cirrhosis	1 (1)	0 (0)	0 (0)	1 (1)
Traumatic	1 (1)	0 (0)	0 (0)	1 (1)
Presence of Hepatocellular carcinoma	21 (28)	0 (0)	0 (0)	21 (23)
Child-Pugh class				
A	42 (56)	3 (38)	7 (70)	52 (56)
B	29 (39)	4 (50)	2 (20)	35 (38)
C	2 (3)	0 (0)	1 (10)	3 (3)
Location of gastric varices				
IGV1	56 (75)	2 (25)	9 (90)	67 (72)
GOV2	19 (25)	6 (75)	1 (10)	26 (28)
Form of gastric varices				
Tumorous	26 (35)	1 (13)	2 (20)	29 (31)
Nodular	49 (65)	7 (88)	8 (80)	64 (69)
Previous gastric variceal bleeding				
Present	36 (48)	4 (50)	2 (20)	42 (45)
Absent	39 (52)	4 (50)	8 (80)	51 (55)
Temporary hemostasis				
Clipping	3 (4)	1 (13)	2 (20)	6 (7)
EVL	5 (7)	1 (13)	0 (0)	6 (7)
Histacryl	3 (4)	1 (13)	0 (0)	4 (4)
Balloon tamponade	2 (3)	0 (0)	0 (0)	2 (2)
Spontaneous	23 (31)	1 (13)	0 (0)	24 (26)
Duration of hospitalization (mean ± SD, d)	17.9 ± 23.4	14.6 ± 12.8	10.4 ± 4.0	16.8 ± 21.4
Follow-up (mean ± SD, d)	1040 ± 713	600 ± 381	704 ± 389	966 ± 677
Range (d)	35 - 2805	1134 - 150	83 - 1183	35 - 2805

BRTO : Balloon occluded retrograde transvenous obliteration; PTO : Percutaneous transhepatic obliteration; Com-T : Combined therapy; EVL : Endoscopic variceal ligation; IGV1: Isolated gastric varices; GOV2 : Gastroesophageal varices.

transvenous obliteration (BRTO) and percutaneous transhepatic obliteration (PTO) therapy, and the clinical efficacy, the complications and outcomes of this therapy were investigated. The aim of this retrospective study was to evaluate the effect of interventional embolotherapy, in particular the clinical efficacy of combined therapy, for the treatment of patients with gastric fundal varices.

MATERIALS AND METHODS

Patients

From 1995 to 2004, 93 patients with gastric fundal varices underwent interventional radiologic embolotherapy at our hospital. The algorithmic strategy used for the treatment of gastric fundal varices in our hospital is shown in Figure 1. According to the treatment algorithm, all the patients were first treated with the BRTO procedure. When the BRTO procedure could not obliterate the gastric varices because of collateral venous drainage, the PTO procedure was chosen as the second line of treatment. When neither the BRTO nor the PTO procedure could obliterate the gastric varices, the patients were treated with a combination of BRTO and PTO as the third line of treatment. Of the 93 patients, 75 were treated with the BRTO procedure; 8 were with the PTO procedure; and 10 were with the combined therapy. The characteristics of the 93 patients are shown in Table 1. The study included 47 men and 46 women with a mean age of 63 years. Table 1 shows the underlying cause of portal hypertension, the presence of serum hepatitis virus markers, and modified Child's classification of the patients[11]. Left-side portal hypertension due to splenic vein obstruction after traumatic pancreatitis was observed in 1 patient. Patients with Child's class C cirrhosis with ascites were excluded from this study because interventional radiologic embolotherapy may increase the portal pressure[12]. Of the 93 patients, 37 (40%) underwent treatment of esophageal varices prior to treatment of gastric varices. 21 (23%) had complications due to hepatocellular carcinoma (HCC). Computed tomography (CT) or ultrasonography could be used to exclude the presence of portal tumor thrombus in all patients who had HCC. Patients with portal vein thrombus were excluded.

According to the classification system originally proposed by Sarin *et al*[13,14], gastric fundal varices are classified as type 2 gastroesophageal varices (GOV2; often long and tortuous, extending from the esophagus below the gastroesophageal junction toward the fundus) or type 1 isolated gastric varices (IGV1; varices located in the fundus that are often tortuous and complex in shape). In our study, 67(72%) patients showed IGV1 and 26 (28%) showed GOV2. The form of fundal varices was massively tumorous in 29 (31%) patients and nodular in 64 (69%).

Gastric fundal varices with active bleeding (spurting or oozing) or signs of recent bleeding or those that were in danger of rupturing were identified for treatment. Variceal bleeding was endoscopically confirmed in all patients immediately after their hematemesis. The bleeding episodes were attributed to ruptured gastric varices based on one of the following criteria: actively bleeding varices at the time of endoscopic examination, presence of adherent clots on varices, or presence of blood in the stomach with gastric varices as the only possible cause of the bleeding. The indications for gastric varices embolization in patients who have not previously had gastric variceal bleeding are as follows: gastric varices with red spots observed during upper intestinal endoscopy or growing gastric varices were considered to be in danger of rupturing.

Bleeding gastric varices were observed in 42 patients, and the remaining 51 patients were prophylactic nonbleeding cases that were in danger of rupturing. An emergency procedure was performed in 14 patients who had active bleeding during urgent endoscopy. To achieve temporary hemostasis, gastric variceal bleeding was treated in the following manner: endoscopic variceal ligation (EVL) in 6 patients, endoscopic injection sclerotherapy (EIS) using cyanoacrylate with lipiodol in 4 patients, clipping treatment in 6 patients, and balloon tamponade in 2 patients. Spontaneous hemostasis was achieved in the remaining 24 patients. Written informed consent was obtained from all patients.

BRTO procedure

Under local anesthesia, a 6.5-French occlusive balloon catheter with a diameter of 20 mm (Create Medic, Yokohama, Japan) was inserted through the femoral or internal jugular vein into the gastrorenal shunt, gastrocaval shunt, or both. Results of balloon-occluded retrograde transvenous varicerography (BRTV) were obtained in advance. Following identification of the gastric varices and their associated feeding and draining veins, the balloon was inflated with CO_2. When the gastric varices were visualized and retention of the contrast medium in the gastric varices was identified, a 50 g/L ethanolamine oleate (EO) solution with iopamidol (50 g/L EOI), which contained equal amounts of 100 g/L EO (Grelan, Tokyo, Japan) and iopamidol 300 (Schering, Berlin, Germany), was used as a sclerosant; the solution was slowly infused through the catheter into the gastric varices and their feeding veins in a retrograde manner during balloon occlusion under fluoroscopy. When varices and feeding veins could be shown in their entirety, injection was suspended. If draining minor collaterals were visualized by retrograde transvenous varicerography, microcoils were used in addition to 50 g/L EOI to embolize the collateral veins in order to prevent leakage of the sclerosing agent into the systemic circulation. After the sclerosant had remained in place for 1 h to allow thrombi formation, hemolyzed blood and excess uncoagulated sclerosant were collected through the catheter; subsequently, the balloon was deflated. Immediately prior to the treatment, 4000 U of haptoglobin (Welfide, Osaka, Japan) was administered by drop infusion to prevent renal damage induced by hemolysis due to the use of EO. The PTO procedure was chosen another day later on when retrograde venography showed that retention of the contrast medium was insufficient even though the collateral vessels had been embolized using metallic coils. (Figure 2A)

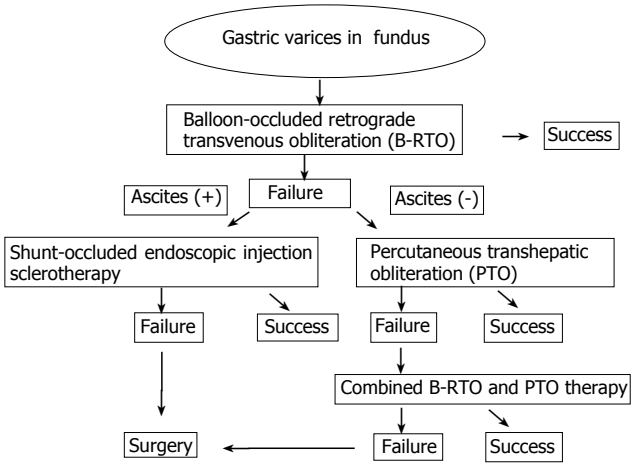


Figure 1 The algorithmic strategy for the treatment of gastric varices.

PTO procedure

We believe that the basic goal of embolotherapy in the treatment of gastric varices is to obliterate the variceal lumen and induce submucosal sclerosis. Therefore, in our hospital, PTO is used to obliterate gastric varices by using a sclerosing agent (50 g/L EOI); this procedure differs from the PTO procedure used previously[15]. Percutaneous transhepatic portography (PTP) using a 5-French catheter with a diameter of 11 mm was performed to determine the hemodynamics of the feeding and draining veins of the gastric varices. Due to the presence of multiple feeding veins, a coaxial catheter was inserted into these feeding veins. Some metallic coils were placed in these feeding veins with the exception of one feeding vein. The balloon catheter was selectively inserted into the remaining feeding vein. Through the balloon catheter, antegrade venography was performed with the balloon inflated. When the gastric varices were visualized and retention of the contrast medium in the gastric varices was identified, 50 g/L EOI, as a sclerosant, was slowly injected in the antegrade direction into the gastric varices under fluoroscopy. When varices could be shown in their entirety, injection was suspended. After the sclerosant had remained in place for 1 h to allow thrombi formation, the hemolyzed blood and excess uncoagulated sclerosant were collected through the catheter. Then, the microcoils were used to embolize the remaining feeding vein and to stabilize the thrombus

in the gastric varices. Portography was repeated to assess obliteration of the feeding veins and the absence of flow to the gastric varices. Immediately prior to the treatment, 4000 U of haptoglobin was administered by drop infusion. When antegrade venography showed that retention of the contrast medium in the gastric varices was insufficient, combined therapy was chosen (Figure 2B).

Combined BRTO and PTO therapy

The BRTO and PTO procedures were performed simultaneously. In other words, the feeding and the draining veins of gastric varices were simultaneously obliterated by the balloon catheters. Through the balloon catheter in the feeding vein, antegrade venography was performed with the double balloon inflated. When the gastric varices were visualized and retention of the contrast medium in the gastric varices was identified, 50 g/L EOI, as a sclerosant, was slowly injected in the antegrade direction into the gastric varices under fluoroscopy. When varices could be shown in their entirety, injection was suspended. After the sclerosant had remained in place for 1 h to allow thrombi formation, the hemolyzed blood and excess uncoagulated sclerosant were collected through the catheter. Immediately prior to the treatment, 4000 U of haptoglobin was administered by drop infusion (Figure 2C).

Evaluation of efficacy of the procedure and follow-up

A follow-up evaluation was performed to check for recurrence and rebleeding of the gastric varices, the aggravation of esophageal varices, complications, and rates of survival in each procedure group. CT was performed 1 wk after the procedure to evaluate obliteration of the gastric varices. Technical success was defined as complete clotting of the gastric varices that were observed during contrast-enhanced CT scanning 1 wk after the treatment. For patients in whom considerable blood flow remained, interventional embolotherapy was repeated until disappearance of blood flow within the gastric varices was confirmed. Initially, endoscopic examination was performed after 1 wk, 3 and 6 mo of the procedure to evaluate the obliteration of the gastric varices. Later, the examination was performed after every 6 mo or whenever clinically required. The laboratory data, including hepatic and renal function tests, were analyzed after 1 d, 1 wk, 1 and 6 mo of the procedure. When red spots on the esophageal varices were detected during endoscopy, the varices were considered to have aggravated and were treated endoscopically as soon as possible. The survival follow-up period was measured as the number of days from the date when interventional embolotherapy was performed until the date of the patient's death or the most recent clinical visit.

Statistical analysis

The cumulative survival rate and the nontreatment rate of esophageal varices were calculated using the Kaplan-Meier method, and the values were compared by means of the Wilcoxon signed rank test. Statistical software (Statview version 5.0; SAS Institute, North Carolina, USA) was used for statistical analysis. $P < 0.05$ was considered statistically significant.

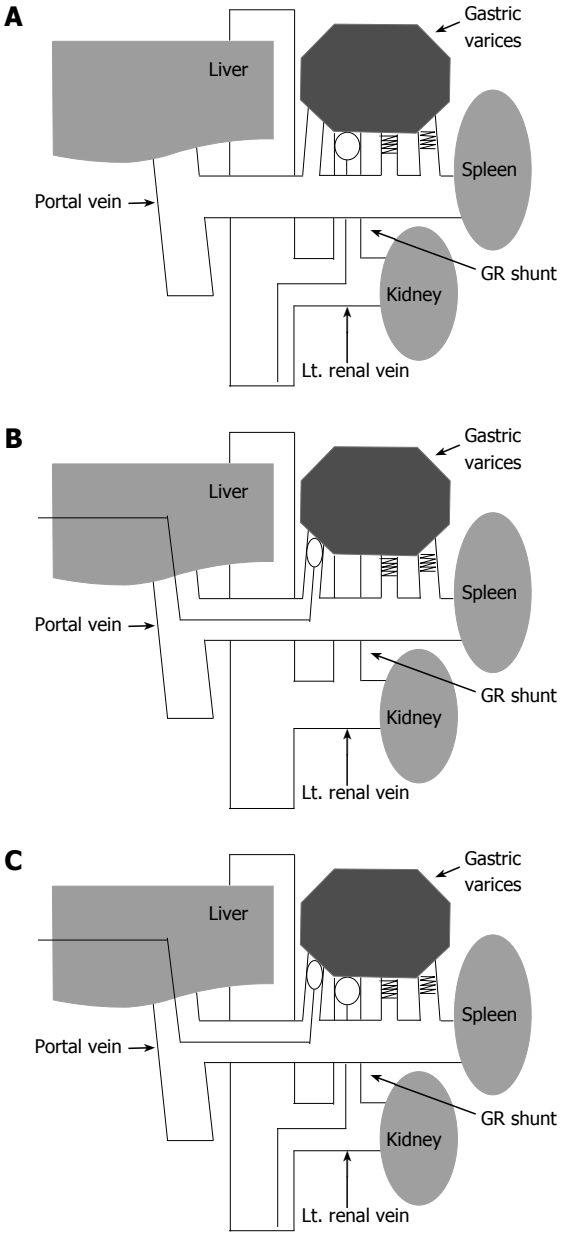


Figure 2 Illustration of radiologic embolotherapy for gastric varices. **A**: Balloon-occluded retrograde transvenous obliteration (BRTO); **B**: percutaneous transhepatic obliteration (PTO); **C**: combined BRTO and PTO therapy; GR = gastrorenal.

RESULTS

The mean observation period was 966 ± 677 d. The mean duration of hospitalization for the treatment of the gastric varices was 17 d. According to the treatment algorithm, the BRTO, PTO, and combined therapy were angiographically successful in 81% (75/93), 44% (8/18), and 100% (10/10) patients, respectively. The three-tiered treatment strategy used in this study enabled successful treatment of all the gastric fundal varices. In 68 of the 75 (91%) patients in the BRTO group, the gastric varices were completely thrombosed; this was shown by the CT that was performed 1 wk after the initial BRTO. The remaining

Table 2 Results of 93 patients treated with radiologic embolotherapy for gastric varices *n* (%)

Results	BRTO	PTO	Com-T	Total
Complete disappearance of gastric varices	75	8	10	93
Variceal rebleeding	1 (1)	1 (13)	0	2 (2)
Recurrence of gastric varices	3 (4)	3 (38)	0	6 (7)
Aggravation of esophageal varices	27 (36)	3 (38)	3 (30)	33 (36)
Death	21 (28)	0	2 (30)	23 (25)
Cause of death				
Hepatic failure	8 (11)	0	1 (10)	9 (10)
Hepatocellular carcinoma	8 (11)	0	0	8 (9)
Cerebral hemorrhage	2 (3)	0	0	2 (2)
Renal failure	2 (3)	0	0	2 (2)
Pneumonia	1 (1)	0	0	1 (1)
Psychosis	0	0	1 (10)	1 (1)

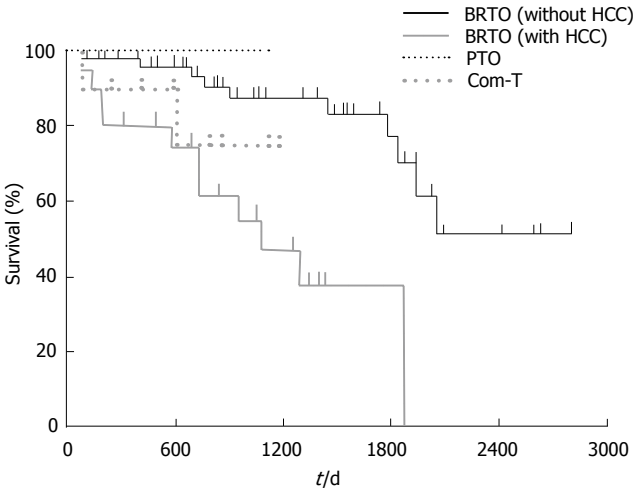


Figure 3 Cumulative survivals after radiologic embolotherapy for gastric varices.

7 (9%) patients who showed no improvement in the gastric varices, as indicated by CT, underwent an additional BRTO procedure. The BRTO procedure was performed twice on 5 patients, and three times on 2 patients with large gastric varices and dilated collateral veins. The overall BRTO technical success rate was 89% (68/75 + 5/7 + 2/2 = 75/84). In 18 patients who underwent variceal embolization by PTO alone or combined with BRTO, CT examination revealed that total obliteration of the gastric varices was achieved in all patients 1 wk after the initial procedure. According to the definition of technical success, the success rate was 91%, 100% and 100% in the BRTO, PTO and combined therapy.

During the follow-up period, recurrence of gastric varices was found in 3 patients (4%) in the BRTO group (1939, 1067, and 723 d after the BRTO procedure) and in 3 patients (38%) in the PTO group (1123, 574, and 150 d after the PTO procedure). In 3 patients in the BRTO group, the GOV2 type of recurrent gastric varices occurred after treatment of IGV1 with the BRTO procedure. Among the 3 patients in the PTO group, 2 had gastric varices of the GOV2 type, while 1 had gastric varices of the IGV1 type before the PTO procedure. In these 3 patients, the type of recurrent gastric varices was identical to those found prior to the PTO treatment. These recurrent gastric varices were strictly followed up without additional treatment. No variceal recurrence was found in the combined therapy group (Table 2).

Rebleeding was observed in 2 patients after treatment (1 patient had received the BRTO procedure and the other had received the PTO treatment). In 1 patient in the BRTO group, rebleeding from gastric varices was observed before the additional BRTO procedure because of incomplete obliteration of the varices by the initial treatment. The gastric varices of the patient were obliterated by the second BRTO procedure. In 1 of the 3 patients who had been noted to have recurrent gastric varices in the PTO group, the rebleeding occurred due to the recurrence. The patient refused additional interventional embolotherapy and underwent Hassab's devascularization after initial hemostasis by an endoscopic procedure[16]. In the combined group, no rebleeding was found.

After the BRTO procedure, the 1- and 3-year survival rates were 92% and 75%, respectively. The 1- and 3-year survival rates were 98% and 87% in the patients without HCC in the BRTO group, 100% and 100% in the PTO group, and 90% and 75% in the combined therapy group, respectively. There was no significant difference across the three groups. On the other hand, the 1- and 3-year survival rates were 80% and 47% in the patients with HCC in the BRTO group. The survival rate after the BRTO procedure was significantly higher in the patients without HCC than in those with HCC ($P < 0.01$, Figure 3).

During the follow-up period, 21 patients in the BRTO group and 2 patients in the combined therapy group died; however, no patients in the PTO group died. Death due to hepatic failure occurred in the case of 8 (11%) patients in the BRTO group (range, 91-2056 d; median, 743 d) and 1 (10%) patient in the combined therapy group (605 d after the procedure). No significant difference was observed among the three procedures. The other causes of death in the BRTO group were hepatocellular carcinoma, cerebral hemorrhage, renal failure (due to diabetes mellitus), and pneumonia. In the combined therapy group, 1 patient died from psychosis 83 d after the procedure (Table 2).

All three procedures caused adverse effects, such as fever (> 38.0℃), hemoglobinuria, abdominal pain, chest pain, nausea, and blood pressure elevation. On the other hand, ascites, pleural effusion, headache, and hepatic encephalopathy were detected in the BRTO group. No fatal complications were observed (Table 3). We have reported the change in the hepatic and renal function tests in patients treated with the BRTO procedure[17]. In terms of laboratory data, there was no difference in the three procedures. The esophageal varices aggravated in 27 (36%) patients in the BRTO group and in 3 patients

Table 3 Complication of treatment *n* (%)

Complication	BRTO	PTO	Com-T	Total
Fever	47 (63)	4 (50)	9 (90)	60 (65)
Hemoglobinuria	35 (47)	3 (38)	10 (100)	48 (52)
Abdominal pain	48 (64)	6 (75)	7 (70)	66 (71)
Nausea	31 (41)	4 (50)	6 (60)	41 (44)
Blood pressure elevation[1]	22 (29)	2 (25)	4 (40)	28 (30)
Ascites	3 (4)	0	0	3 (3)
Pleural effusion	5 (7)	0	0	5 (5)
Headache	6 (8)	0	0	6 (7)
Hepatic encephalopathy	1 (1)	0	0	1 (1)
Chest pain	24 (32)	2 (25)	7 (70)	33 (36)

[1]More than 20 mmHg elevation.

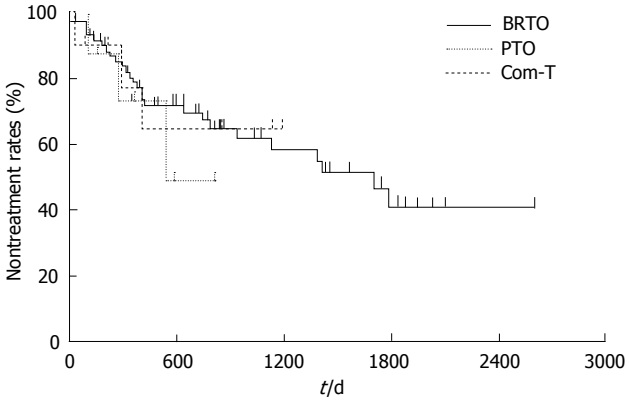


Figure 4 Cumulative non-treatment rates for esophageal varices after radiologic embolotherapy.

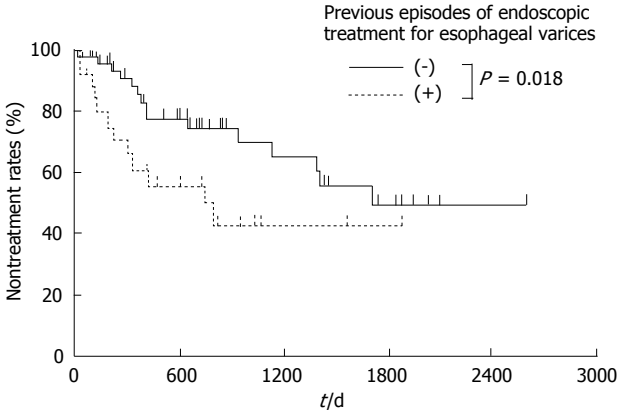


Figure 5 Cumulative non-treatment rates for esophageal varices after balloon-occluded retrograde transvenous obliteration.

in each of the PTO (38%) and combined therapy groups (33%). These were treated using the usual methods of EVL and EIS. During the course after the procedures, the cumulative nontreatment rates of esophageal varices at 1 year were 79%, 73%, and 77% in the BRTO, PTO, and combined therapy groups, respectively. There was no significant difference in the three procedures (Figure 4). The nontreatment rates of esophageal varices in the BRTO group at 1 and 3 year were 61% and 42% in the patients with esophageal varices and 86% and 70% in the patients without them prior to BRTO, respectively. The cumulative nontreatment rates of esophageal varices in the BRTO group were significantly lower in the patients with esophageal varices than in the patients without them prior to BRTO ($P < 0.01$, Figure 5).

DISCUSSION

Since Kanagawa *et al*[18] reported the treatment of gastric varices by BRTO with 50 g/L EOI, BRTO has been widely accepted as an interventional embolotherapy for gastric varices in Japan. Subsequently, many reports demonstrated the clinical efficacy and safety of this treatment[6-9]. According to several reports, the therapeutic result obtained by this treatment is satisfactory and the recurrence rate is very low. In our study, the BRTO procedure was angiographically successful in 81% (75/93) patients; hence, our results are consistent with those reported previously[9,10,19]. Recurrence of gastric varices was found in only 3 of the 75 patients who had undergone the BRTO procedure. In all 3 patients, the GOV2 type of recurrent gastric varices occurred after treatment of IGV1 with the BRTO procedure. Gastric varices that are classified as GOV2 have a direct connection with the coronary vein and esophageal varices. The aggravation of esophageal varices caused by damage of portal hemodynamics after the BRTO procedure has been well documented. It appeared that the mechanism of these recurrent gastric varices was similar to that observed in the aggravation of esophageal varices.

The BRTO procedure is applied to gastric varices that have a portosystemic shunt route to the inferior vena cava. Some gastric varices without a major shunt or those with many small collateral circulations are difficult to treat by the BRTO procedure. Reports of the refractory varices treated with the BRTO procedure are rare[10,20,21]. Thus, an adequate treatment strategy for refractory gastric varices has not yet been established. We performed the PTO procedure for uncontrollable gastric varices treated by the BRTO procedure. The first report on the treatment of gastric varices by the PTO procedure was described by Scott *et al* in 1976[15]. Since the previously used PTO procedure could obliterate inflow vessels but could not easily obliterate gastric varices completely, it was associated with a high incidence of rebleeding and recurrences. Our PTO procedure would fill the lumen of the gastric varices with the sclerotic agent in an antegrade manner by stopping the inflow into varices using coils and a balloon catheter that is wedged in an antegrade manner. EO, an anionic surfactant, infiltrates and destroys the cell membrane of venous endothelial cells. Damage to the venous endothelium caused by EO is greater than that caused by a 50% glucose solution. Therefore, EO causes thrombus formation in gastric varices, and gastric varices thrombosed by EO have been reported to be completely disappeared[6-9]. The PTO procedure that we used is the same as BRTO in that the gastric varices are thrombosed.

In this study, the PTO procedure rescued only 44%

(8/18) patients who had uncontrollable gastric varices treated by the BRTO procedure. To block the gastric variceal blood flow, the PTO procedure, which involves the catheterization of the only feeding vein, may be less effective than the combined therapy. It is probable that the antegrade procedure could obliterate the feeding vein but could not completely and easily obliterate the varices with a large draining vein. The patients in the PTO group had more minor collateral circulation, such as pericardial or hemiazygous veins, as compared with the patients in the BRTO group.

The indication for the PTO procedure with 50 g/dL EOI was the presence of gastric varices without large draining veins and varices untreated by BRTO because of many small collateral veins. In the PTO group, the gastric varices recurred in 3 patients with incomplete eradication; bleeding occurred in 1 of these patients. It appeared that the therapeutic efficacy of the PTO procedure was incomplete for uncontrollable gastric varices treated by the BRTO procedure.

Some of the patients who underwent attempted BRTO had no embolization performed at all *via* antegrade route because of unfavorable anatomy whilst others had some embolization with coils and/or sclerosant. We performed the combined BRTO and PTO therapy on patients in whom uncontrollable gastric varices had been treated by the BRTO procedure and the PTO procedure. Reports on the varices that had been treated with combined therapy, which obstructs both the feeding and the draining veins of the varices, are rare. Kimura *et al*[22] reported a case of rectal varices that was treated with double balloon-occluded embolotherapy in 1997. Similarly, Ota *et al*[23] reported the results of combined therapy comprising BRTO and transileocolic vein obliteration (TIO) in a patient with duodenal varices. Kiyosue *et al*[24] suggested that the indication for combined therapy was the presence of gastric varices that are supplied by single or multiple gastric veins with coexistent gastric veins that are directly contiguous with the shunt but do not contribute to the varices. However, to the best of our knowledge, there have been no studies on the treatment of gastric varices by combined BRTO and PTO therapy. In our study, the therapeutic result of the combined therapy was satisfactory. No bleeding or variceal recurrence was found in the combined therapy group.

Combined therapy, which can obstruct both the feeding and the draining veins of gastric varices to retain the sclerosing agent completely in the varices, may provide a better control of the gastric variceal blood flow than that provided by the only BRTO or PTO procedure. Our study demonstrates that combined therapy is very effective in the treatment of gastric varices, irrespective of whether patients have had a large gastrorenal shunt and/or collateral circulation. Since the combined therapy may eliminate the hemodynamics of gastric varices, this procedure may be more effective than other procedures. In fact, the obliteration of the gastric varices by this method was successful in all 10 patients in whom the BRTO or PTO procedure was unsuccessfully performed.

Although only the femoral or internal jugular vein is punctured in the BRTO procedure, the combined therapy or the PTO procedure requires a puncture in the liver. Therefore, the BRTO procedure is less invasive than the other procedures. Since the combined therapy appears to be similar to the BRTO procedure in terms of safety and efficacy, we propose that the BRTO procedure should be the first choice of the treatment for gastric varices. The combined therapy should only be performed in the case of highly selected patients. We believe that the indication for combined therapy is refractory gastric varices that remain untreated despite being subjected to the BRTO and PTO procedures. In addition, the PTO procedure is less invasive than the combined therapy, because the femoral or internal jugular vein is not punctured in this procedure. Thus, the results presented here indicate that the algorithmic strategy for treating gastric varices using the BRTO and PTO procedures and the combined therapy is feasible (Figure 1).

These three procedures for obliterating the gastric varices may increase the portal pressure. Aggravation of the esophageal by these treatments varices was recognized to be a result of elevated portal pressure. From the viewpoint of aggravation of esophageal varices, no statistical differences were demonstrated among the three procedures. However, non-treatment rate of esophageal varices after the BRTO was significantly lower in those who were treated esophageal varices before BRTO than those not treated. Our results are consistent with those reported earlier[19]. Periodic endoscopic follow-up is recommended after the eradication of gastric varices, particularly in patients with esophageal varices prior to the BRTO procedure. The mortality rate for gastric variceal bleeding is much higher than that for esophageal variceal bleeding[25]. Therefore, we believe that these changes in the portal hemodynamics were preferable because esophageal varices can be easily controlled by endoscopic therapy.

In conclusion, combined BRTO and PTO therapy may rescue cases with uncontrollable gastric fundal varices that remained even after treatment with BRTO and/or PTO. Our study could establish the algorithmic strategy for treating gastric fundal varices using interventional embolotherapy. However, there were limitations of our study, including retrospective nature and discrepancy in sample size between the BRTO, PTO and combined therapy groups. Further, long-term prospective studies would assist in clarifying the effect of combined BRTO and PTO therapy.

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RAPID COMMUNICATION

Family history of irritable bowel syndrome is the major determinant of persistent abdominal complaints in young adults with a history of pediatric recurrent abdominal pain

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Abstract

AIM: To assess the late outcome of teen-agers with a previous history of recurrent abdominal pain (RAP) or irritable bowel syndrome (IBS).

METHODS: A group of 67 children with RAP referred to the department from January 1986 to December 1995 was followed up between 5 and 13 years after the initial diagnosis by means of a structured telephone interview. We hypothesized that those patients with persistent adult IBS-like symptoms would be significantly more likely to report a family history of IBS in comparison with adults with no persistent abdominal complaint.

RESULTS: Out of the 52 trackable subjects, 15 were found to present IBS-like symptoms at follow-up (29%) whereas the majority (37 subjects) did not. Subjects with IBS-like symptoms were almost three times more likely to present at least one sibling with similar symptoms compared to subjects not complaining (40.0% *vs* 16.0%), respectively ($P < 0.05$ at Student t test). Subjects with IBS-like symptoms also reported a higher prevalence of extra-intestinal symptoms, such as back pain, fibromyalgia, headache, fatigue and sleep disturbances.

CONCLUSION: The study confirms previous observations indicating that pediatric RAP can predict later development of IBS. The latter appears to be greatly influenced by intrafamilial aggregation of symptoms, possibly through the learning of a specific illness behavior.

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INTRODUCTION

Recurrent abdominal pain (RAP) is characterized by three or more episodes of abdominal pain that occur over at least three months and are severe enough to interfere with activities, such as school attendance and performance, social activities, and participation in sports and extracurricular activities[1].

RAP is common, affecting between 7% to 25% of school-aged children and adolescents[2,3], and may be responsible for 2% to 4% of pediatric office visits[4]. RAP becomes more prevalent with increasing age into adolescence[1,5-7] and is more common in girls[2,8] with an equal gender ratio in early childhood[3,9], but greater female symptom reporting in late childhood and adolescence[1,7,10]. Specific structural, infectious, inflammatory, or laboratory abnormalities are unusual in RAP, particularly in the absence of "red flags" such as weight loss, gastrointestinal bleeding, pain awakening the child at night, systemic symptoms such as fever, or laboratory evidence of anemia or inflammation[11].

Medically unexplained RAP is often considered to be a functional gastrointestinal disorder (FGD), a condition defined as a variable combination of chronic or recurrent gastrointestinal symptoms in the absence of explanatory structural or biochemical abnormalities[12]. Irritable bowel syndrome (IBS) is a FGD characterized by recurrent abdominal pain or discomfort occurring over a 3-mo period, where the symptoms are relieved by defecation and/or are associated with changes in stool frequency or consistency[13].

Implications of pediatric RAP across the lifespan are not well understood. Early follow-up studies have reported

persistence of recurrent abdominal pain into adulthood for one third to one half of affected children[14-16], but are limited by the lack of standardized assessments and formal diagnostic criteria. In more recent studies using standardized assessments and control groups, former RAP patients reported significantly greater abdominal pain, other somatic symptoms, functional impairment, health service use, and internalizing psychiatric symptoms in comparison to controls at 5-year follow-up, and females with a history of RAP are significantly more likely to meet diagnostic criteria for IBS[17,18]. A large, population-based cohort study of adults born in 1946 in the United Kingdom compared the 2% of the sample who consistently reported medically unexplained abdominal pain at ages 7, 11, and 15 years with study participants without a history of chronic abdominal pain on the basis of standardized assessments when participants were 36 years old. Persistent abdominal pain in childhood is significantly associated with an increased risk of psychiatric disorder in adulthood, but not with abdominal pain or headache once psychiatric disorder is controlled for in the regression[19].

In this study, a group of children with RAP or IBS was followed up between 5 and 13 years after the initial diagnosis. We hypothesized that those patients with persistent adult IBS-like symptoms would be significantly more likely to report a family history of IBS in comparison with adults with no persistent abdominal complaint.

MATERIALS AND METHODS

Case identification and recruitment

Potential patients were identified from clinical records of the Pediatric Department at the "L. Sacco" University Hospital of Milan. Charts of children referred to the department from January 1986 to December 1995 were retrieved, searching those children aging between 4 and 14 years at the time of index visit and referred because at least three episodes of medically unexplained abdominal pain occurred and they had an established a diagnosis of RAP or IBS. Criteria for inclusion included: Those proposed for RAP by Apley & Naish in 1958 and revised by Hyams JS *et al*[6], e.g. at least 3 episodes of medically unexplained abdominal pain occurring during a period of at least 3 months that were severe enough to affect the activities of the child or those proposed for IBS by the Rome II Working Party[13], e.g. in children old enough to provide an accurate pain history, at least 12 wk, even not consecutive, in the preceding 12 mo of abdominal discomfort or pain with two out of the following three features: relieved with defecation, and/or onset associated with a change in frequency of stool, and/or a change in form (appearance) of stool, and no structural or metabolic abnormalities[13].

Criteria for exclusion included: abdominal pain with atypical features, symptoms, or findings suggestive of physical disease (e.g. abnormal physical examination or laboratory findings, persistent vomiting, gastrointestinal bleeding, constitutional symptoms such as fever or weight loss), acute or chronic physical disease, and development disability. There were 67 patients identified who fulfilled the diagnostic criteria for either RAP or IBS.

An effort to contact by telephone the potential participants to the study was made, at an interval between 5 and 13 years after the index visit. However, 13 patients could not be tracked, because of change of address or available number incorrect, 1 because already deceased, and 1 because of interview refusal. Thus, data were available for 52 subjects (contact rate: 88%), with only 1 subject declining to participate (refusal rate: 1.5%).

Table 1 Demographic and other clinical features of individuals with or without IBS at follow-up

	IBS symptoms ($n = 15$)	No IBS ($n = 37$)	*P* value
Mean age (yrs) at index diagnosis	7.7	6.2	> 0.05
Female/male	9/6	19/18	> 0.05
Mean duration of follow-up (yr)	8.4	8.6	> 0.05
Siblings with IBS (%)	40	16	< 0.05
Extra-intestinal symptoms (%)	47	13.5	< 0.05
Body mass index	19.9	18.9	> 0.05

The telephone interview was done by two of us (*ZG* and *DGS*) unaware of the index diagnosis of the subjects. A structured questionnaire was used, developed for the purpose of the study, asking very simple questions, as for example actual weight and height, presence or absence of abdominal complaints, presence or absence of siblings with similar complaints, *etc*. In case of affirmative answer to the question regarding presence of abdominal complaints, the interviewer specifically asked the questions 20 to 27 from the Rome II Modular Questionnaire[20], relating to a better characterization of bowel and abdominal pain symptoms, respectively, translated and linguistically adjusted for Italian people. Finally, the severity of dominant abdominal symptom as well as the impact on work/school/sport activities were assessed by using an 1-100 analogue scale. If one sibling was identified as suffering from abdominal complaints as well, then an effort was made to verify this information by a subsequent direct telephone to him or her, and the same questions were asked in case of confirmation.

No payment or other revenge was offered to subjects who agreed to participate in the study.

RESULTS

Fifteen subjects were found to present IBS-like symptoms at follow-up (29%) whereas the majority (37 subjects) did not. The demographics and other clinical features of IBS patients and non-patients at follow-up are presented in Table 1.

Subjects with IBS-like symptoms were almost three times more likely to present at least one sibling with similar symptoms compared to subjects not complaining (40.0% *vs* 16.0%), respectively ($P < 0.05$ at Student *t* test). Not surprisingly, subjects with IBS-like symptoms reported a higher prevalence of extra-intestinal symptoms, such as back pain, fibromyalgia, headache, fatigue and sleep disturbances.

Table 2 shows the features of the 15 subjects with IBS-like symptoms in more detail. Two thirds of the patients reported abdominal pain as opposed by one third only re-

Table 2 Clinical features of the15 subjects with IBS-like symptoms at follow-up

Symptoms	%
Predominant abdominal pain	67
Predominant abdominal discomfort	33
Alternating diarrhea/constipation	15
Severity of dominant symptom (1-100)	50
Limitation in school/sport/play (1-100)	48
Sleep disturbances	19
Headache	15

porting abdominal discomfort. Approximately half of the patients (53%) reported diarrhea as the main bowel habit abnormality, 32% constipation and 15% an alternating habit, respectively.

The predominant symptom was scored by the subjects as moderate severity (50% severity at the analogue scale) and judged to be the cause of significant limitation in daily activities (48% at the analogue scale). Finally, the more frequent extra-intestinal symptom reported was the presence of sleep disturbances (19%), followed by headache (15%).

DISCUSSION

The major finding of our study is the observation that a considerable proportion of young adults (29%), who suffered from a RAP (or IBS) during their childhood severe enough to be referred to the pediatric department of our hospital, still continue to suffer from abdominal complaints who fulfill the diagnostic criteria for IBS. This finding is in agreement with previous published works, in particular the recent papers by Hotopf *et al*[19], Walker *et al*[18], and Campo *et al*[21]. Hotopf *et al*[19] did a population-based birth cohort study, assessing the cohort of 3637 individuals born in UK in one week of 1946. These subjects were prospectively evaluated by means of several "waves" of data gathering, in particular at the ages of 7, 11, and 15 years the information was sought concerning abdominal pain. By doing so, the authors were able to diagnose "persistent" abdominal pain as abdominal pain reported at each of these three time-points, suggesting that pain is chronic. They showed that at the age of seven, 20% of survey members were suffering from abdominal pain, at the age of eleven they were 19% and at the age of fifteen they were 17%, whereas at the age of 36 years, only 7% of the subjects still had persistent abdominal pain. Abdominal symptoms in childhood appear to be weakly associated with persistent abdominal pain or headache in adulthood, but are associated with increasing numbers of physical symptoms at this age[19].

In the study by Walker *et al*[18], 76 patients with RAP and 49 control age- and sex-matched subjects, suffering from acute minor illness or injury, were compared 5 years after their initial evaluation by a structured telephone interview. The subjects constituted a higher proportion of the initial samples, e.g. 84% and 94%, respectively. It turned out that at follow-up frequent (weekly or more often) abdominal pain was significantly more likely to be reported by patients with a history of RAP (45%) than by control subjects (20%) ($P < 0.01$). Patients with a history of RAP were also more likely to report having received a diagnosis of IBS in the 5 years since study entry (5% *vs* 0%). Finally, the former subjects had significantly more school absences and clinical visits because of abdominal pain than the control subjects, while similar levels of school absence and clinical visits for non abdominal symptoms were reported.

Finally, the recent paper by Campo *et al*[21] compared two small samples of young adults, i.e. 28 subjects were evaluated for RAP between the age of 6 and 17 and 28 matched controls participating at the same age in a study about tonsillectomy and adenoidectomy, an average of 11.1 years after the index visit. In this follow-up study, although approximately one third of the RAP group met the criteria for IBS, patients and controls did not differ significantly on interview measures of IBS (33.3% *vs* 22.2%, NS). One third of RAP patients endorsed a history of migraine, over twice as many as controls, with a trend toward statistical significance (35.7% *vs* 14.3%, $P = 0.07$). The second interesting feature is the familial aggregation of symptoms. In our study, the presence of at least one sibling with IBS was identified in the group developing adult IBS symptoms in a proportion greater than twice that observed in the group not developing adult IBS (40% *vs* 16%, $P < 0.01$). This observation is in keeping with the more recent literature, suggesting the importance of parental reinforcement and modeling in the development of gastrointestinal illness behavior in children and adults. Whitehead *et al*[22] demonstrated that people with a functional bowel disorder are more likely to report being given gifts or treat foods when they had minor illnesses (cold or influenza) as a child. Levy *et al*[23] in a case-control study conducted on 631 children of parents with a recent diagnosis of IBS and 646 children matched by parental age, gender and number of children in the family without this diagnosis, found that case children have significantly more yearly visits than controls for gastrointestinal symptoms (0.35 *vs* 0.18, $P < 0.001$) as well as for all causes (mean 12.26 *vs* 9.81, $P = 0.0001$), suggesting an intergenerational transmission of gastrointestinal illness behavior. Finally, Levy *et al*[24] further explored the relative contribution of genetic and environmental (social learning) influences to the development of IBS by comparing concordance rates in monozygotic and dizygotic twins to concordance between mothers and their children. They found that concordance for IBS is significantly greater in monozygotic than in dizygotic twins (17.2% *vs* 8.4%, $P = 0.03$), supporting a genetic contribution to IBS. In addition, they also found that the proportion of dizygotic twins with IBS who have mothers with IBS is greater than the proportion of dizygotic twins with IBS who have co-twins with IBS (15.2% *vs* 6.7%, $P < 0.001$), suggesting that social learning has an equal or greater influence than heredity.

In our study we did not individually assess the relative contribution of these two factors to abdominal illness behavior, nor measured the influence of this abnormal learned sick role on school or work absenteism, but were able to explore the subjective impact of IBS complaints on one aspect of health-related quality of life, i.e. the limitation in school/sport or playing activities. We found

that, on a scale from 0 to 100 it weighted 49.2. The last finding we would like to emphasize is the relatively low rate (less than 20%) of extra-intestinal symptoms, such as sleep disturbances or headache, in the group of subjects developing IBS. This finding points toward a very specific type of learned illness behavior in our group of patients, again in keeping with the observation of others, in particular the group of Chapel Hill[25], who demonstrated that reinforcement of menstrual illness behavior *or* cold illness behavior in parents significantly predicts adult menstrual *vs* cold symptoms and disability days, independent of stress and neuroticism.

Our study has a number of potential limitations. Like most studies of RAP, this was a referred sample from a specialized care setting, which can limit the generalization of our findings. We did not assess the psychopathology of the subjects nor the history of maltreatment, since this was not formally done at the time the subjects were visited the outpatient department because of RAP. Finally, our study was not a case-control one, but merely a follow-up investigation of a cohort of children. We cannot exclude therefore that the rate of adult IBS development observed in our sample is different from that expected in a matched control population. However, to measure the risk of IBS was not our primary aim, which was in fact to compare the individual and family findings of young adults developing or not developing IBS 5 to 13 years after the original diagnosis of RAP or children IBS. On the other hand, valuable features of our study are the large sample investigated (52 subjects tracked compared to 28 in the study by Campo *et al*[21], the average 8.5 years of follow-up compared to 5 years in the study by Walker *et al*[18], and the use of validated diagnostic criteria for the diagnosis of RAP and IBS).

In conclusion, a large proportion, roughly one third of children referred to the hospital because of RAP or IBS, still presents significant abdominal complaints and can be diagnosed as suffering from IBS 5 to 13 years after this diagnosis, confirming that although RAP is a functional disorder, it should not be dismissed as a completely benign condition or a transient reaction to stress. Furthermore, by comparing young adults presenting or not presenting IBS, the presence of one sibling with IBS is the most important factor associated with IBS development, confirming that functional bowel disorders tend to run in families due to heredity factors and social learning influences.

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RAPID COMMUNICATION

Overexpression of *c-met* in the early stage of pancreatic carcinogenesis; altered expression is not sufficient for progression from chronic pancreatitis to pancreatic cancer

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Abstract

AIM: To investigate *c-met* expression during early pancreatic carcinogenesis.

METHODS: We used 46 bulk tissues and 36 microdissected samples, including normal pancreas, chronic pancreatitis, and pancreatic cancer, for quantitative real-time reverse transcription-polymerase chain reaction.

RESULTS: In bulk tissue analyses, pancreatic cancer tissues expressed significantly higher levels of *c-met* than did chronic pancreatitis and normal pancreas tissues. *c-met* levels did not differ between chronic pancreatitis and normal pancreas tissues. In microdissection-based analyses, *c-met* was expressed at higher levels in microdissected pancreatic cancer cells and pancreatitis-affected epithelial cells than in normal ductal epithelial cells (both, $P < 0.01$). Interestingly, pancreatitis-affected epithelial cells expressed levels of *c-met* similar to those of pancreatic cancer cells.

CONCLUSION: Overexpression of *c-met* occurs during the early stage of pancreatic carcinogenesis, and a single alteration of *c-met* expression is not sufficient for progression of chronic pancreatitis-affected epithelial cells to pancreatic cancer cells.

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Key words: *c-met*; Pancreatic cancer; Chronic pancreatitis; Pancreatic carcinogenesis

Yu J, Ohuchida K, Mizumoto K, Ishikawa N, Ogura Y, Yamada D, Egami T, Fujita H, Ohashi S, Nagai E, Tanaka M. Overexpression of *c-met* in the early stage of pancreatic carcinogenesis; altered expression is not sufficient for progression from chronic pancreatitis to pancreatic cancer. *World J Gastroenterol* 2006; 12(24): 3878-3882

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INTRODUCTION

Pancreatic cancer is the fourth and fifth leading cause of cancer death in the United States and Japan, respectively, and has the lowest survival rate of any solid cancer[1-3]. Because the lack of specific symptoms in patients with pancreatic cancer makes early diagnosis difficult, initial diagnosis typically occurs when the tumor has reached an advanced stage[4]. Therefore, we need a better understanding of the early carcinogenesis of pancreatic cancer to facilitate early detection.

The protooncogene *c-met* encodes a member of the family of receptor tyrosine kinases that is a 190-ku glycoprotein comprised of a transmembrane 145-ku β subunit and an extracellular 50-ku α subunit[5,6]. The met receptor binds to and is activated by hepatocyte growth factor/scatter factor (HGF/SF)[7], leading to increased proliferation, altered motility, and enhanced invasion[8]. The wild-type *c-met* gene is amplified or overexpressed in many types of human cancer, including cancers of the breast, stomach, liver, endometrium, nasopharynx, and pancreas[9-14].

Pancreatic ductal cells, which give rise to the most common type of human pancreatic carcinoma, are particularly sensitive to inflammatory and carcinogenic processes[15]. There are reports that pancreatic inflammation may play a key role in early pancreatic carcinogenesis[16]. Rivera *et al* found that *k-ras* mutations that lead to uncontrolled cell growth and may be the principal molecular event in the pathogenesis of pancreatic cancer are present in chronic pancreatitis, providing a genetic basis for the potential progression of chronic pancreatitis to pancreatic cancer[15]. Taken together, the data indicate that the molecular changes associated with chronic pancreatitis

should be studied as a part of a comprehensive strategy to understand pancreatic carcinogenesis. It was reported that *c-met* mRNA expression was increased during development of chronic pancreatitis in dibutyltin-treated mice[17]. Furukawa *et al* also reported that strong immunostaining of c-Met was present in 58% of specimens demonstrating pancreatic hyperplastic epithelia and in 78% of specimens demonstrating ductal adenocarcinoma, respectively[18]. To understand the clinical significance of *c-met* expression in pancreatic carcinogenesis, we need to examine *c-met* expression during early pancreatic carcinogenesis. Therefore, we need accurate measurements of the levels of *c-met* expression in specific cells, such as normal epithelial cells, pancreatitis-affected epithelial cells, and invasive ductal carcinoma (IDC) cells.

The microdissection method, which can isolate specific cells from a frozen section, is used for genetic analysis of specific lesions[19]. Because tumor cells typically represent only 60%-70% of the cells in pancreatic cancer bulk tissues and because the percentage of chronic pancreatitis-affected epithelial cells is very low in bulk chronic pancreatitis tissues[19], cell microdissection is necessary and useful for reliable molecular analyses related to pancreatic carcinogenesis.

In the current study, we examined *c-met* mRNA levels in 46 human pancreatic bulk tissue samples and cells microdissected from 36 samples of pancreas, including normal pancreas, chronic pancreatitis, and pancreatic cancer by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Our goals were to quantify *c-met* expression in chronic pancreatitis, which is an early stage of pancreatic carcinogenesis, and to clarify when overexpression of c-met occurs during pancreatic carcinogenesis.

MATERIALS AND METHODS

Pancreatic tissues

Tissue samples were obtained at the time of surgery at Kyushu University Hospital (Fukuoka, Japan) as described previously[20]. In brief, tissue samples were removed as soon as possible after resection and divided into at least three bulk tissue specimens. The first sample was embedded in OCT compound (Sakura Findek, Tokyo, Japan), snap-frozen for microdissection, and stored at -80℃ until use. The second sample was fixed in formalin, embedded in paraffin, and cut into 4-μm-thick sections for hematoxylin and eosin (H&E) staining. The third sample was snap-frozen for bulk tissue analysis and stored at -80℃ until use. Tissues adjacent to the specimens were examined histologically, and the diagnosis was confirmed by pathologists. Thirteen pancreatic cancer tissue specimens were obtained from tumoral lesions of resected pancreas with primary pancreatic cancer. Twelve normal pancreatic and 11 pancreatitis-affected pancreatic tissue specimens were taken from peripheral tissues away from the tumor or pancreas resected due to mass-forming pancreatitis. Written informed consent was obtained from all patients, and the study was approved by our institution's surveillance committee and conducted according to the Helsinki Declaration.

Table 1 *c-met* and *β-actin* primer sequences and product size

Primer	Forward	Reverse	Product size
	Sequence 5′-3′	Sequence 5′-3′	
c-met	tgatgatgaggtggacaca	ctatggcaaggagcaaaga	149
β-actin	aaatctggcaccacaccttc	ggggtgttgaaggtctcaaa	139

Pancreatic cancer cell lines

Fourteen pancreatic cancer cell lines, ASPC-1, BxPC-3, KP-1N, KP-2, Panc-1, Suit-2 (provided by Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), MIA PaCa-2, NOR-P1 (established in our laboratory), Capan-1, Capan-2, CFPAC-1, H48N, HS766T, and SW1990 (American Type Culture Collection, Manassas, Virginia), and four primary cultured pancreatic fibroblasts derived from resected pancreatic tumors were used. Cells were maintained as described previously[21].

RNA Isolation

Total RNA was extracted from bulk tissues with an RNeasy Mini Kit (Qiagen, Tokyo, Japan) per the manufacturer's protocol. Total RNA was extracted from cells isolated by microdissection with the standard acid guanidinium thiocyanate-phenol-chloroform protocol[22] with or without glycogen (Funakoshi, Tokyo, Japan).

Quantitative analysis of c-met mRNA expression by real-time RT-PCR

Quantitative real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) with a LightCycler Quick System 350S (Roche Applied Science, Mannheim, Germany) as described previously[23]. In brief, the reaction mixture was first incubated at 50℃ for 15 min to allow for reverse transcription. PCR was initiated with one cycle of 95℃ for 10 min to activate modified Taq polymerase followed by 45 cycles of 94℃ for 15 s, 55℃ for 20 s, and 72℃ for 10 s, and one cycle of 95℃ for 0 s, 65℃ for 15 s, and + 0.1℃/s to 95℃ for melting analysis. Each sample was run twice. In addition, any sample showing more than 10% deviation in the values was tested a third time. The 10% deviation was calculated from the concentrations determined from the calibration curve. The level of *c-met* mRNA expression was calculated from a standard curve constructed with total RNA from the Capan-1 pancreatic cancer cell line. The range of threshold cycles was from 20-35 cycles for *c-met* primers[24] and from 5-30 cycles for *β-actin* primers[25] (Table 1). Expression of *c-met* mRNA was normalized to that of *β-actin* mRNA.

Microdissection-based quantitative analysis of c-met mRNA

Frozen tissues were cut into 8-μm-thick sections. One section was stained with H&E for histologic examination. IDC cells from 13 sections, pancreatitis-affected epithelial cells from 12 sections, and normal ductal epithelial cells from 11 sections were selectively isolated with a laser microdissection and pressure catapulting system (P.A.L.M. Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocols. After microdis-

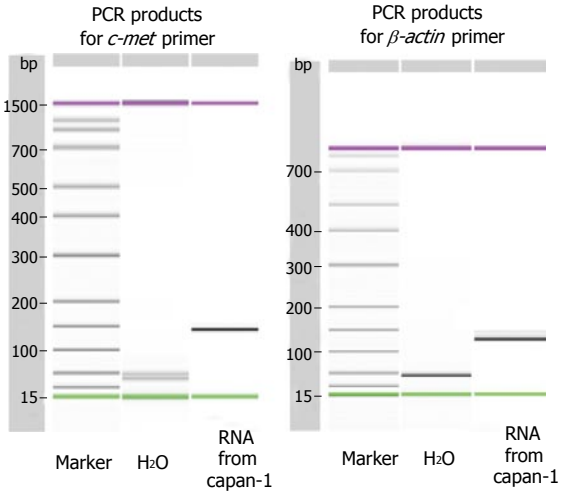


Figure 1 PCR products were analyzed with an Agilent Bioanalyzer 2001. A single 149-bp band was observed for *c-met* primer pairs (left panel). A single 138-bp band was observed for *β-actin* primer pairs (right panel). Each primer pair used in the present study produced a single melting peak on real-time RT-PCR and a single prominent band of the expected size on microchip electrophoresis.

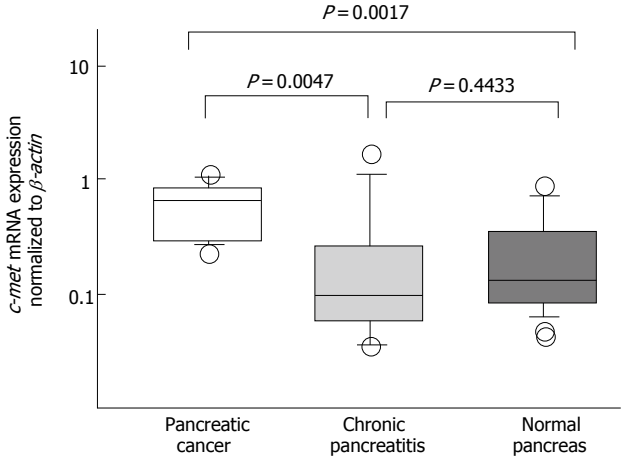


Figure 2 We performed quantitative real-time RT-PCR to quantify *c-met* expression in pancreatic cancer tissues (n = 11), normal pancreatic tissues (n = 20), and chronic pancreatitis tissues (n = 15). *c-met* was overexpressed in pancreatic cancer tissues in comparison to expression in normal pancreatic (P = 0.0017) and chronic pancreatitis tissues (P = 0.0047). Levels of *c-met* did not differ between normal pancreatic and chronic pancreatitis tissues (P = 0.4433).

section, total RNA was extracted from the selected cells and subjected to real-time RT-PCR for quantitative measurement of *c-met* as described previously[19].

PCR products sized by the Agilent 2100 Bioanalyzer

Microchip electrophoresis was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). A DNA 1000 Assay Kit was purchased from Agilent Technologies. As shown in Figure 1, each PCR product was analyzed with DNA 1000 Lab Chips (Agilent Technologies) per the manufacturer's protocol[26].

Statistical analysis

Data were analyzed with the Kruskal-Wallis test for comparison of three groups and Mann-Whitney U test for comparison of two groups because normal distribution was not obtained after logarithmic transformation. Statistical significance was defined as $P < 0.05$. Because we performed multiple comparisons of our real-time RT-PCR data, we conservatively used the Bonferroni correction, and therefore, the adjusted significance level was $P < 0.017$.

RESULTS

Quantitative analyses of c-met expression in bulk pancreatic tissues

In the bulk tissue analyses, we measured *c-met* expression in pancreatic cancer tissues (n = 11), normal pancreatic tissues (n = 20), and chronic pancreatitis tissues (n = 15). As shown in Figure 2, the level of *c-met* expression in normal pancreatic tissues was similar to that in chronic pancreatitis tissues. The median values were 0.130 for normal pancreatic tissues and 0.107 for chronic pancreatitis tissues (P = 0.44). The median value of *c-met* expression in pancreatic cancer tissues was 0.678, which was approximately 5-fold greater than that in normal pancreatic tissues (P = 0.0017) and 6-fold greater than that in chronic pancreatitis tissues (P = 0.0047). All data from bulk tissue analyses indicated that *c-met* was overexpressed in pancreatic cancer, but not chronic pancreatitis tissues.

Quantitative Analysis of c-met expression in 14 pancreatic cancer cell lines and 4 primary pancreatic fibroblast cultures

To confirm expression of *c-met* mRNA in pancreatic cancer cell lines, total RNA was isolated from 14 pancreatic cancer cell lines. As shown in Figure 3, *c-met* was expressed in all 14 pancreatic cancer cell lines with median value of 0.742. The 4 primary cultures of normal pancreatic fibroblast1, 2, 3 and 4 (Panc-f1, Panc-f2, Panc-f3, and Panc-f4) expressed low levels of *c-met* mRNA with a median value of 0.023. Pancreatic cancer and chronic pancreatitis tissues usually contain abundant stromal cells, such as fibroblasts. Most of the bulk pancreatic cancer tissues in the present study also contained many desmoplastic changes and the chronic pancreatitis tissues contained stromal components. Therefore, the level of *c-met* expression detected in bulk tissue analyses may not represent the true levels of expression by specific cells due to dilution of these specific cells by contaminating cells such as stromal fibroblasts.

Quantitative analysis of c-met in microdissected pancreatic cancer cells, pancreatitis-related epithelial cells, and normal pancreatic epithelial cells

In general, bulk pancreatic tissue is complex, containing ductal epithelial cells, acinar cells, fibroblasts, islet cells, and mesenchymal cells. Tumor cells comprise only 60%-70% of the cells in bulk tissue specimens of pancreatic cancer[19]. Therefore, data from bulk tissue analyses may not accurately reflect *c-met* levels in specific cells, such as pancreatic cancer cells, pancreatitis-affected epithelial cells, and normal ductal epithelial cells. It has been reported that *c-met* is expressed in acini and pancreatic islets in normal human pancreas[27]. To avoid the influence of contami-

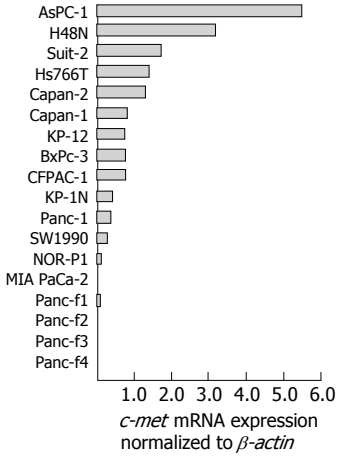


Figure 3 *c-met* expression in pancreatic cancer cell lines and primary pancreatic fibroblasts. The median value of *c-met* expression from pancreatic cancer cell lines was 0.742. In contrast, the median value of *c-met* expression in 4 primary cultures of pancreatic fibroblasts (Panc-f1, Panc-f2, Panc-f3, and Panc-f4) was 0.023.

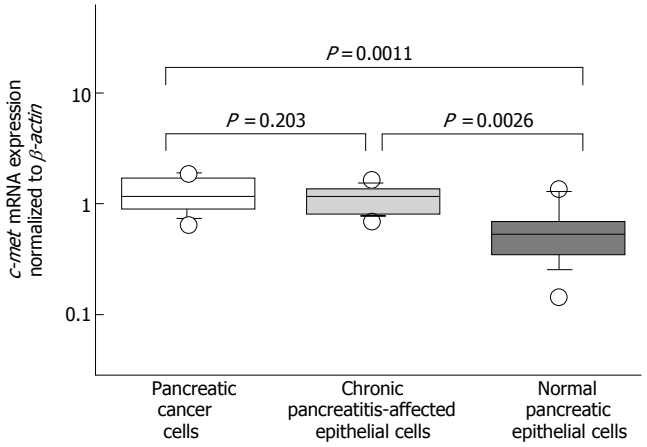


Figure 4 Quantitative analysis of *c-met* mRNA levels in microdissected pancreatic cancer cells (n = 13), pancreatitis-affected epithelial cells (n = 11), and normal pancreatic ductal epithelial cells (n = 12). *c-met* levels in pancreatic cancer cells (median, 1.208) were 2.21-fold higher than those in normal ductal epithelial cells (median, 0.546; P = 0.0011). *c-met* levels in pancreatitis-affected epithelial cells (median, 1.211) were 2.22-fold higher than those in normal ductal epithelial cells (median, 0.546; P = 0.005). *c-met* levels in microdissected normal ductal epithelial cells were the lowest with a median value of 0.546. Interestingly, pancreatitis-affected epithelial cells expressed levels of *c-met* that approached those in pancreatic cancer cells.

nating non-ductal cells, we used a laser-microdissection method to select specific cells for analysis. For microdissection analyses, we isolated pancreatic cancer cells from 13 sections, normal pancreatic ductal epithelial cells from 12 sections, and pancreatitis-affected epithelial cells from 11 sections. As shown in Figure 4, *c-met* expression in pancreatic cancer cells (median, 1.208) was 2.21-fold higher than that in normal pancreatic duct epithelial cells (median, 0.546; P = 0.0011). *c-met* level in normal pancreatic ductal epithelial cells was the lowest. Interestingly, the *c-met* level in pancreatitis-affected epithelial cells (median, 1.211) was significantly higher than that in normal pancreatic duct epithelial cells (median, 0.546; P = 0.005). These data suggested that *c-met* was overexpressed in chronic pancreatitis-affected epithelial cells at levels close to those in pancreatic cancer cells.

DISCUSSION

We performed quantitative real-time RT-PCR to measure *c-met* expression in pancreatitis-affected epithelial cells, which may progress to pancreatic cancer[28-30], to clarify the significance of *c-met* expression in an early stage of pancreatic carcinogenesis. Bulk tissue analyses revealed that *c-met* was overexpressed in pancreatic cancer, but *c-met* expression did not differ between chronic pancreatitis and normal pancreatic tissues. In microdissection-based analyses, we found that pancreatitis-affected epithelial cells expressed high levels of *c-met* that approached those of pancreatic cancer cells. Although it may be difficult to make comparison because microdissection data are based on c-met mRNA levels in single cell, our findings appear to be inconsistent with those of previous immunohistochemical studies in which the positive rates of c-Met were 58%, 80%, and 78% of specimens demonstrating hyperplastic epithelia, severely dysplastic epithelia, and pancreatic ductal adenocarcinoma, respectively[18]. However, Furukawa *et al*[18] also reported that pancreatic cancer patients with diffuse c-Met immunostaining survived longer than c-Met-negative patients. Welm *et al* reported that overexpression of c-*Met* alone did not result in development of tumors, although c-Met acted cooperatively with other genetic alterations, such as overexpression of *MYC*, to induce mammary tumorigenesis[31]. In addition, there have been several reports that the cumulative risk of pancreatic cancer among patients with chronic pancreatitis is only 1% to 4%[28-30]. Taken together, these data suggest that overexpression of *c-met* occurs in the early stage of pancreatic carcinogenesis but is not sufficient for progression of chronic pancreatitis-affected epithelial cells to pancreatic cancer cells. However, these data also suggest that pancreatitis-affected epithelial cells expressing high levels of *c-met* in conjunction with other genetic or epigenetic changes may have the potential to progress to pancreatic cancer. Therefore, *c-met* may be a useful marker for identifying persons with high-risk lesions that may progress to pancreatic cancer.

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RAPID COMMUNICATION

Influence of a microscopic positive proximal margin in the treatment of gastric adenocarcinoma of the cardia

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Abstract

AIM: To investigate the influence of a positive proximal margin in total gastrectomy patients with gastric adenocarcinoma of the cardia.

METHODS: Medical records of 191 patients with total gastrectomies for adenocarcinoma of the cardia between 1995 and 2000 were reviewed. The clinicopathologic features associated with a positive margin were determined, and the predictors for survival were analyzed.

RESULTS: The incidence of positive proximal margin was 8.4% (16/191). The positive margins were associated with advanced diseases. The tumor size and the depth of tumor invasion were independent risk factors for a positive margin. The mean survival in the positive margin group was 33.9 mo as compared with 62.4 mo in the negative group ($P < 0.001$). However, the difference in survival lost significance in subgroup analysis according to stage. Multivariate analysis identified that a positive margin was not an independent prognostic factor for survival.

CONCLUSION: A positive margin is more of an indication of advanced disease in patients with gastric adenocarcinoma of the cardia rather than an independent prognostic factor for survival.

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Key words: Resection margin; Gastric cancer; Total gastrectomy; Outcome

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INTRODUCTION

A curative resection is the only treatment offering hope for cure in patients with gastric cancer. This suggests removing the primary lesion with an adequate tumor-free margin[1,2]. Several reports have looked into the detrimental effect of a positive margin on survival in gastric cancer patients[3-5]. However, these studies have included heterogeneous groups with respect to the resection margins. Patients with various tumor locations of gastric cancer or with proximal and distal margins of various types of gastrectomies are grouped together.

Gastric cancer of the cardia has been regarded as a separate neoplasm because of its distinct prognostic and pathological features[6]. A positive proximal resection margin poses a management dilemma for surgeons. To our knowledge, there are few reports on the influence of a positive proximal margin in total gastrectomy for adenocarcinoma of the cardia, and there is some controversy as to whether a positive margin is an independent prognostic factor for survival or not.

The aim of this retrospective study was to investigate the risk factors associated with a positive proximal margin in gastric adenocarcinoma of the cardia, and to assess the influence of a positive margin on the patient's long-term survival, as well as its independent impact on prognosis.

MATERIALS AND METHODS

Medical records were reviewed of 202 patients with adenocarcinoma of the cardia undergone surgery between September 1995 and December 2000 at the Department of Surgery, Yonsei University College of Medicine. Two patients with recurrent gastric cancer, 2 with preoperative metastatic findings and 7 with incomplete data were excluded from the analysis. Therefore, 191 patients who underwent total gastrectomy with curative intent for gastric adenocarcinoma of the cardia, were enrolled in the study. One hundred and twenty-nine patients (67.5%) were males and 62 patients were females. The median age was 57 years ranging from 29 to 85 years.

Table 1 Comparison of demographic and pathologic data between patients with positive and negative margins *n* (%)

Variable	Positive margins (*n* = 16)	Negative margins (*n* = 175)	*P* value
Age (years)	49.3 ± 12.7	57.5 ± 11.2	0.006
Sex			0.161
Male	8 (50.0)	121 (69.1)	
Female	8 (50.0)	54 (30.9)	
Siewert type			0.141
Type II	5 (31.3)	25 (14.3)	
Type III	11 (68.7)	150 (85.7)	
Gross type			0.001
Borrmann I, II	1 (6.3)	83 (47.4)	
Borrmann III, IV	15 (93.7)	92 (52.6)	
Tumor size (cm)			0.001
≤ 5 cm	2 (12.5)	98 (56.0)	
> 5 cm	14 (87.5)	77 (44.0)	
Histology			0.399
Differentiated	3 (18.8)	56 (32.0)	
Undifferentiated	13 (81.2)	119 (68.0)	
LRM (cm)			0.985
≤2	4 (25)	42 (24.0)	
> 2 and ≤ 4	10 (62.5)	113 (64.6)	
> 4	2 (12.5)	20 (11.4)	

LRM: length of resection margin. Age represents mean ± SD.

Table 2 Comparison of tumor, node, metastasis classification, stage between patients with positive and negative margins *n* (%)

Variable	Positive margins (*n* = 16)	Negative margins (*n* = 175)	*P* value
Depth of tumor invasion			< 0.001
T1	0 (0)	29 (16.6)	
T2	2 (12.5)	84 (48.0)	
T3	11 (68.7)	55 (31.4)	
T4	3 (18.8)	7 (4.0)	
Node involvement			< 0.001
N0	3 (18.8)	67 (38.3)	
N1	2 (12.5)	60 (34.3)	
N2	2 (12.5)	26 (14.8)	
N3	9 (56.2)	22 (12.6)	
Stage			< 0.001
I	0 (0)	57 (32.6)	
II	1 (6.3)	42 (24.0)	
III	6 (37.5)	51 (29.1)	
IV	9 (56.2)	25 (14.3)	

Table 3 Logistic regression analysis of risk factors for a positive margin

Variable	Regression coefficient	Standard error	Odds ratio	95% CI for odds ratio	*P* value
Serosal invasion	2.300	0.782	9.970	2.152 - 46.196	0.003
Tumor size	1.875	0.787	6.524	1.395 - 30.512	0.017

The location of tumor and preoperative staging were determined by esophagoduodenogastroscopy, upper gastrointestinal barium examination and abdominal CT scan. All patients had either a type II or III tumor according to the Siewert classifications[7]. A total gastrectomy with transhiatal resection of the distal esophagus and esophagojejunostomy were performed and a macroscopically tumor free proximal resection margin was achieved on all patients. Intraoperative frozen section margin evaluation was not routinely carried out except in patients with suspicion of obtaining a positive margin and thus was not analyzed. During the operation, one hundred and twenty-five of these patients (65.4%) underwent splenectomy for the purpose of lymph node dissection or suspicion of direct tumor invasion to the spleen. A D2 lymphadenectomy was regularly performed. In our institute, postoperative fluorouracil-based adjuvant chemotherapy was usually given in advanced cancer patients except for those with T2N0M0 stage.

A resection margin was considered positive if the permanent section examination revealed cancer infiltration at the line of the transection. The length of the resection margin was defined as the distance from the proximal limit of the lesion to the site of the resection. Collected data included patient demographics, clinicopathologic features and survivals. Stage was reported according to the fifth version of the UICC/AJCC guidelines. Survival was measured from the date of operation to death or to the last follow-up date of June 30, 2003. Of the 191 patients, 88 (46.1%) died during the follow-up period.

Statistical analyses were performed using SPSS version 10.0 (SPSS, Chicago, IL, USA). Univariate analysis was done using two-tailed χ^2 test or unpaired Student's *t*-test. The risk factors associated with a positive margin were identified using a logistic regression model. Survival probability was estimated using the Kaplan-Meier method. The survival curves were compared using the log-rank test. The prognostic factors for survival were analyzed using the Cox proportional hazards regression model with forward stepwise method. $P < 0.05$ was considered statistically significant.

RESULTS

Sixteen out of the 191 patients (8.4%) had a positive margin. The incidence of this event was 26.5% (9/34) in patients with stage IV gastric cancer, while 10.5% (6/57) in stage III and 2.3% (1/43) in stage II. None of the 57 patients with stage I disease had a positive margin. The mean length of the proximal resection margin was 2.5 ± 1.1 cm (range, 0.8-7.0 cm). It was 2.6 ± 1.4 cm (range, 0.8-6.5 cm) in the positive group as compared with 2.5 ± 1.2 cm (range, 0.8-7.0 cm) in the negative margin group (P = 0.703). A positive margin was significantly associated with Borrmann III and IV gross type, large size of the primary tumor, serosal invasion, node involvement and advanced stage disease (Table 1, Table 2). Logistic regression analysis revealed that the size of the primary tumor and the depth of tumor invasion were independent risk factors for a positive margin (Table 3).

The mean follow-up time and survival time after surgery were 44.3 mo (range, 0.5-93.5 mo) and 59.9 mo (95% confidence interval, 54.6-65.1 mo), respectively.

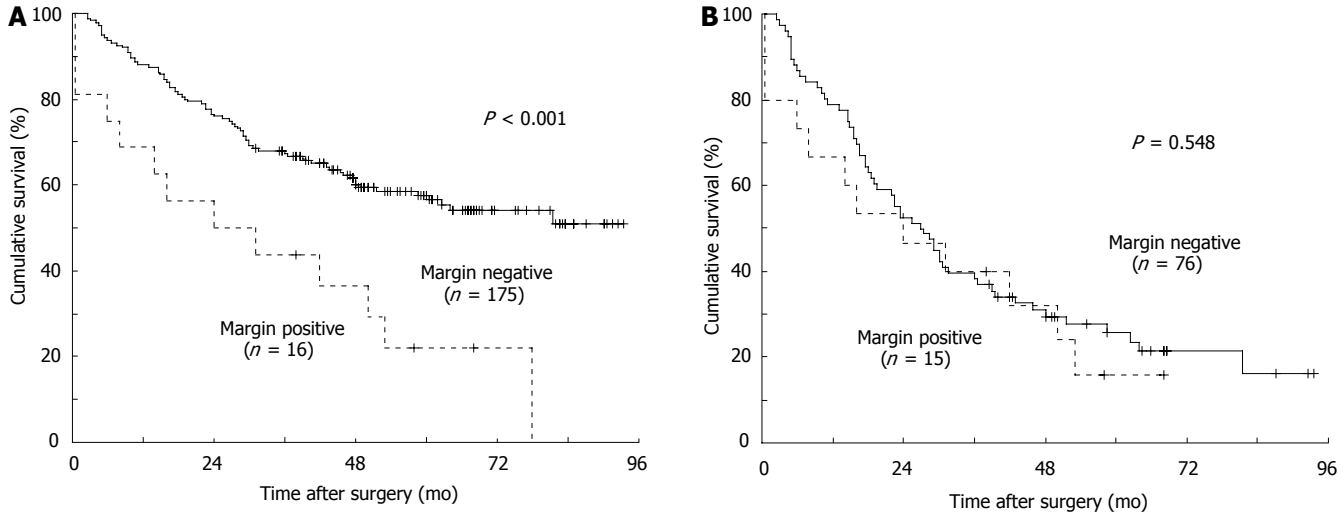


Figure 1 Cumulative survival curves according to the margin status in a total of 191 patients (**A**) and 91 stage III and IV (**B**) patients with gastric cancer of the cardia. The mean survival time of patients with positive margins was 33.9 mo compared to 62.4 mo of patients with negative margins in total 191 patients, and 29.2 mo compared to 38.2 mo in 91 patients with stage III and IV diseases

On univariate analysis, the gross type, size of the primary tumor, serosal invasion, lymph node involvement, stage, resection margin status and splenectomy were significantly associated with the long-term survival. The mean survival time in patients with positive margins was 33.9 mo as compared with 62.4 mo in patients with negative margins ($P < 0.001$, Figure 1A). Since fifteen out of 16 patients (93.8%) with positive margins had stage III and IV gastric cancer in the present study, we then analyzed the subgroup patients stratified by stage, the difference in survival between both groups did not reach significance in stage III and IV gastric cancer (Figure 1B).

On multivariate analysis, seven variables with a significance level of < 0.05 in univariate analysis were included in the model. Positive margin was not identified to be an independent predictor for survival (Table 4).

Table 4 Multivariate analysis of prognostic factors for survival

Variable	Regression coefficient	Standard error	Odds ratio	95% CI for odds ratio	*P* value
Gross type	-0.174	0.258	0.840	0.507 - 1.392	0.500
Tumor size	0.209	0.248	1.232	0.758 - 2.001	0.399
Serosal invasion	-0.125	0.264	0.883	0.526 - 1.481	0.637
Node involvement	0.838	0.431	2.311	0.994 - 5.374	0.052
Stage	1.469	0.389	4.344	2.028 - 9.304	< 0.001
Margin status	0.397	0.330	1.488	0.779 - 2.843	0.229
Splenectomy	0.276	0.292	1.318	0.744 - 2.337	0.344

DISCUSSION

The incidence of positive resection margin has been reported to vary from 5% to 35%[8-10]. In this study, the incidence of this event was 8.4%. However, it was significantly higher in patients with stage III and IV gastric cancer (16.5%) than in patients with stage I and II gastric cancer (1.0%). There are several factors accounting for such a high incidence of positive margin in advanced gastric cancer patients. First, it has been reported that the incidence of positive margin increases with deeper invasion of the tumor into the gastric wall and advanced disease stage[11,12]. Secondly, patients with stage III and IV disease have more Borrmann III and IV gross type of tumors and/or more undifferentiated tumors (data not shown), and these patients may have a higher risk of sub-clinical intramural tumor infiltration to the esophagus[10]. Finally, intraoperative frozen section margin evaluation is not routinely performed in advanced disease patients, which may possibly increase the incidence of a positive margin in those patients[13].

The risk factors for a positive margin in the present study were identified based on the same length of the proximal resection margin between margin positive and negative groups. Logistic regression analysis showed that the risk mainly depended on the depth of cancer invasion and the size of the primary tumor.

The depth of invasion into the gastric wall is significantly related to a higher rate of esophageal infiltration. Bozzetti *et al*[14] reported that the risk of proximal infiltration is significantly higher when the tumor infiltrates the serosa or beyond it. Songun *et al*[14] demonstrated that the depth of tumor invasion is one of the independent prognostic factors for a positive margin in gastric cancer at various tumor locations. Our results are similar to the aforementioned reports. The size of the primary tumor was also identified as an independent predictor for a positive margin, which is in agreement with that reported by Yokoda *et al*[16], who found that a lesion exceeding 5 cm in maximum diameter is predisposed to a positive margin in gastric cancer.

Although frozen section has a high sensitivity and specificity, it is not routinely used to assess the resection margin[3,17,18]. It is advisable that this technique should be used selectively for patients who may benefit from it since frozen sections are costly and time-consuming[19]. Our study observed that patients with advanced stage disease had a higher risk of obtaining a positive margin. In addition, a

positive margin was associated with deeper tumor invasion to the gastric wall and larger tumor size. These findings may provide some evidence for helping the surgeons select patients to perform intraoperative frozen section margin evaluation.

The influence of a positive margin on prognosis of gastric cancer of the cardia remains controversial. Chan *et al*[20] reviewed 137 total or proximal gastrectomies for gastric cancer with various lesion sites and observed that a positive oesophageal margin is an independent poor prognostic factor for long-term survival. A similar result has been reported by Mariette *et al*[21], who reviewed 94 adenocarcinomas of the esophagogastric junction, but different surgical approaches were involved in their work. In addition, there are no difference in the clinicopathologic features between the positive margin group and the negative margin group in the study[21]. These aforementioned studies are not in accordance with the results we observed that a positive margin was not associated with the long-term survival in gastric adenocarcinoma of the cardia. This discrepancy, although has to be interpreted with caution, may be explained by the following reasons. First, it was a retrospective study and the analyses were based on relatively few patients with or without positive margins in our single institution. Second, a positive margin is often associated with advanced disease. Gall *et al*[22] investigated 87 patients who underwent esophagectomy for cancer and found that the positive margin is involved mainly in late stage disease which does not influence the long-term survival in those patients. Kim *et al*[23] reported that a positive margin loses its predictive value for survival in patients with T3 or T4 lesions and stage III or IV disease. Finally, because the relationship between margin status and treatment failure patterns were not addressed in our study with the limited data, our results may possibly due to the uncertainty in advanced disease, the patients may usually succumb to metastatic disease before the effect of the positive margin is observed[24].

Our results suggest that a positive margin is more of an indication of advanced disease rather than an independent prognostic factor for survival in patients with gastric cancer of the cardia. However, more definitive conclusions would be based on multi-center studies with the results of large-scale analyses.

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RAPID COMMUNICATION

Laparoscopic cholecystectomy: A report from a single center

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Abstract

AIM: To review and evaluate our experience in laparoscopic cholecystectomy.

METHODS: A retrospective analysis was performed on data collected during a 13-year period (1992-2005) from 1220 patients who underwent laparoscopic cholecystectomy.

RESULTS: Mortality rate was 0%. The overall morbidity rate was 5.08% (n = 62), with the most serious complications arising from injuries to the biliary tree and the cystic artery. In 23 (1.88%) cases, cholecystectomy could not be completed laparoscopically and the operation was converted to an open procedure. Though the patients were scheduled as day-surgery cases, the average duration of hospital stay was 2.29 d, as the complicated cases with prolonged hospital stay were included in the calculation.

CONCLUSION: Laparoscopic cholecystectomy is a safe, minimally invasive technique with favorable results for the patient.

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Key words: Laparoscopy; Cholecystectomy; Minimally invasive

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INTRODUCTION

Laparoscopic cholecystectomy was first introduced by Muhe in 1986[1], and has now evolved to the point where it has replaced the open technique in many medical centers around the world. Today, laparoscopic cholecystectomy, rather than the open technique, is considered as the treatment of choice for gallstone disease[2-5].

Perceived advantages of laparoscopic cholecystectomy, compared with the open technique, include earlier return of bowel motility, less post-operative pain, better cosmetic result and shorter hospital stay resulting in equal or lower hospital costs, as documented by various randomized control trials[6-9].

At the university hospital of Patras, we performed our first laparoscopic cholecystectomy on September 1992. Since then, a total of 1220 laparoscopic cholecystectomies have been performed. As our surgeons are increasingly familiar with it, laparoscopic cholecystectomy has become the most common operation in our daily practice.

MATERIALS AND METHODS

Subjects

This is a retrospective study that involves data collection from medical records of patients undergoing laparoscopic cholecystectomy. Data collection for this research project was approved by the Ethics Committee. The Department of Surgery at the University Hospital of Patras maintains medical records for all patients who have an operation in this institution since the hospital opened in 1988. Our records include paper-only charts up to 1999, and electronic records since then. The records of all patients who underwent laparoscopic cholecystectomy between September 1992 and September 2005 in our institution were retrieved. During this period, a total of 1220 patients underwent laparoscopic cholecystectomy. The female to male ratio was 3:1 (907 *vs* 313) and the mean patient age was 53.3 (range 16-86) years.

Data collection

Data recorded included demographic information, past medical history, diagnosis and indication for the operation, name of surgeon and anesthesiologist, duration of the operation, peri-operative and post-operative complications, time to return of bowel function, oral intake and ambulation, postoperative pain and hence further need for analgesics and length of hospital stay. The recorded perioperative complications included subcutaneous

emphysema, pneumothorax, bleeding, hemodynamic instability, myocardial ischemia, injury to the biliary tree or the cystic artery.

Postoperative complications included re-operation, ileus, fever, atelectasis, pneumonia, wound infection, incisional hernia, myocardial ischemia or infarction, unplanned ICU admission or death. Data were entered into a computerized database for further analysis. Most patients (86.3%) presented uncomplicated biliary colic. The remainder had symptoms of acute cholecystitis, pancreatitis and cholangitis. All patients had an ultrasound of the upper right abdominal quadrant and, in selected cases, an ERCP was performed before the operation.

All patients scheduled for elective cholecystectomy were admitted the day before, and were allowed to have food and liquids until 6 h before the operation.

Surgical technique

The operation was performed with the standard four-port technique, using carbon dioxide for peritoneal cavity insufflation. For the first 283 cases, the Veres technique was used to obtain pneumoperitoneum. Thereafter and for the subsequent cases, we preferred the Hasson technique. Following gallbladder removal, drains were placed selectively in difficult cases where there was a risk of postoperative bleeding. All patients wore anti-embolic stockings and received low molecular heparin subcutaneously. Antibiotic prophylaxis was ensured with 3 peri-operative doses of first generation cephalosporin intravenously. The administration of heparin continued postoperatively, until the discharge of the patient from the hospital. The average time of the operation was 72 (range 50-102) min.

Anesthetic management

All procedures were performed under general endotracheal anesthesia. The anesthetic regimen was not standardized, but was tailored to the needs of each patient, as determined by the attending anesthesiologist. All patients were NPO (nil per os) after midnight, or, for cases done later in the day, for at least 6 h before the operation. All medications, except anti-platelet agents or anticoagulants, were continued until the morning of surgery. Before induction of anesthesia, patients were premedicated with intravenous ranitidine 50 mg, and midazolam 2-5 mg, depending on age, gender and preoperative anxiety. Most patients received an antiemetic (metoclopramide or ondansentron) to prevent post-operative nausea and/or vomiting. Anesthesia was induced with intravenous administration of 50-100 μg of fentanyl, followed by intravenous propofol (2 mg/kg) or thiopental (3-5 mg/kg). Muscle relaxation was accomplished with a non-depolarizing relaxant (vecuronium 0.1 mg/kg or rocuronium 0.5 mg/kg). After intubation, anesthesia was maintained, in most cases, with inhaled agents (isoflurane, sevoflurane or desflurane in an air/oxygen mixture), although TIVA with opioids and propofol infusion at 100-200 μg/kg per min was occasionally used. After its introduction to the market, remifentanil was widely used, combined with propofol or an inhaled agent, to provide intra-operative analgesia. Analgesia was accomplished with intravenous fentanyl in the early part of the procedure, followed by intravenous or intramuscular meperidine (25-50 mg) towards the end. In addition, patients who exhibited severe pain also received NSAID as an adjuvant analgesic. At the end of the procedure, all port sites were injected with 5 g/L bupivacaine (total of 10 mL). Additional meperidine or morphine was given in the recovery room if required, based on patient VAS pain score. All patients received an intravenous infusion of 1-2 L of crystalloid solution during the operation. Patients who could not tolerate pneumoperitoneum, or in cases where urine output was less than 0.5 mL/kg per hour, additional colloid (60 g/L hetastarch 500 mL) was administered, to ensure adequate patient hydration.

Post-operative analgesia was achieved with acetaminophen (*po*, 500 mg 4 times a day), and oral codeine/acetaminophen combinations (30-60 mg 4 times daily). Additional metoclopramide or ondansentron was administered if nausea or vomiting persisted postoperatively. All patients had oral liquids and were encouraged to proceed with food in the evening after the operation, provided there was no nausea or vomiting.

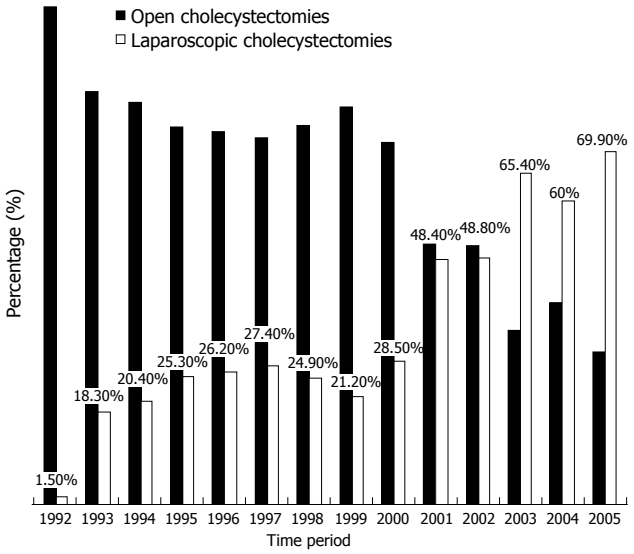


Figure 1 Laparoscopic *vs* Open cholecystectomies over the time period.

RESULTS

Today laparoscopic cholecystectomy has become the golden rule for the treatment of gallstone disease. In our clinic, the laparoscopic approach was initially reserved for the uncomplicated cases. However, as experience was accumulated and our surgeons became more familiar with the technique, even more complicated cases were treated laparoscopically. In 1994, 18.1% of all cholecystectomy procedures (53 cases) in our institutions were performed laparoscopically. This percentage kept increasing every year, to the point where in 2004, 60% (148 patients) of all cholecystectomies were performed laparoscopically (Figure 1). Prior to operation, 72 patients underwent preoperative ERCP due to ultrasonographic suspicion of

Table 1 Conversion to open cholecystectomy

Reason	*n* (%)
Bleeding	6 (0.49)
Bile leakage	3 (0.24)
Duodenal injury	1 (0.08)
Technical Problem	1 (0.08)
Anatomic Variation (gallbladder aplasia)	1 (0.08)
Inadequate exposure (dense adhesions- inflammation)	11 (0.9)

choledocholithiasis, elevated titer of bilirubin and liver function enzymes, recent history of jaundice and/or pancreatitis. Patients with stones in the common bile duct were treated with endoscopic sphincterotomy.

Mortality rate was 0%, but complications did occur, especially early in our series, when surgical experience was still limited. Concerning major complications, bile leak from the cystic duct occurred in 3 (0.24%) patients. In two cases, the leak resulted from failure to clip the cystic duct due to unsatisfactory application of the endoscopic clips. The problem was recognized before the conclusion of the operation in both cases. However, in one of these cases, ligation was impossible, and conversion to the open procedure was necessary. In a third case, the cystic duct injury went undiagnosed intraoperatively, and was only suspected when a significant amount of bile accumulated in the drain on the 1st postoperative day. In this case ERCP confirmed the diagnosis. Endoscopic sphincterotomy was performed and a naso-biliary drainage tube was placed. The leak eventually resolved without any further therapy after 7 d. In 2 (0.16%) patients, a common bile duct injury occurred. In the first occasion, the common bile duct was clipped as it was falsely believed to be the cystic duct. Unfortunately, that mistake went unrecognized intraoperatively and it was only suspected the next postoperative day when the patient developed obstructive jaundice. Eventually, the diagnosis was confirmed with an ERCP and the patient was operated immediately for end-to-end repair of the duct and T-tube placement. In the second case the patient was released from the hospital after a 24 h stay only to return after 2 d with fever, leucocytosis and signs of peritonitis. After an abdominal CT the diagnosis of choloperitoneum was made and the patient was operated on the same afternoon. Intraoperatively, a leak was identified in the common bile duct and it was attributed to the diathermy. After proper cleaning of the peritoneal cavity, the defect was restored with a T-tube placement. In both patients the end result was favorable and no further manipulation was needed.

In 3 (0.24%) cases, bile leak from the gallbladder bed occurred after removing the gallbladder. One case, in whom the leak was identified intra-operatively, was converted to the open procedure. However, in two other cases, the diagnosis was made post-operatively and the patients required ERCP with sphincterotomy.

Thermal injury of the duodenum (2nd segment) occurred in 1 (0.08%) patient. The injury was handled with conversion to open cholecystectomy and suturing of the traumatic lesion.

Bleeding from injury of the cystic artery occurred in 5 (0.4%) patients, 3 of which were admitted and had surgery emergently for acute cholecystitis. The procedure was converted to open cholecystectomy, and bleeding was controlled successfully in all cases. In 10 (0.81%) cases, the bleeding originated from minor accessory vessels of the gallbladder fossa. In 4 of these patients (2 with history of hepatic cirrhosis and 2 with preexisting coagulation abnormalities), conversion to an open procedure was necessary to control bleeding. In 4 (0.32%) cases, bleeding occurred from the port site, and in 1 of them conversion to open procedure was necessary to achieve homeostasis.

Four (0.32%) patients developed a surgical site infection. In 3 of them, gram (+) bacteria were isolated (*S. aureus* in 2, *Enterococcus spp* in 1), whereas in 1 case *E. coli* was the underlying pathogen. All cases were treated successfully with intravenous antibiotics. However, in one case surgical drainage of the wound was necessary. Twenty-seven (2.21%) patients developed moderate post-operative fever. All cases of fever were considered of respiratory origin and treated as such with good outcome.

Two (0.16%) patients developed subcutaneous emphysema, but this was a transient phenomenon and did not cause any morbidity. Hernias at the trocar sites occurred as late complications in 2 (0.16%) patients.

The reported rate of conversion to open cholecystectomy ranges between 3 and 7%[10,11]. In our series, 23 (1.88%) of all procedures were converted to the open technique (Table 1). In most cases, uncontrollable bleeding and bile leakage, as well as previous abdominal operation associated with dense adhesions were the main reasons for conversion to the open procedure.

Finally, 2 (0.16%) patients returned to the hospital 1 wk after hospital discharge with signs of choledocholithiasis. This was attributed to stones in the biliary tree that went undiagnosed at the time of the operation. Both patients were treated with ERCP and sphincterotomy, resulting in a good outcome.

DISCUSSION

Today, laparoscopic cholecystectomy is considered as the treatment of choice for gallstone disease. In our department, laparoscopic cholecystectomy was initially reserved for otherwise healthy patients with uncomplicated disease. However, as experience was gained, laparoscopic cholecystectomy was also offered for patients with more complicated biliary disease, such as acute cholecystitis, former pancreatitis and choledocholithiasis.

Laparoscopic cholecystectomy confers definite advantages compared to the open procedure, but carries its own risks. Consequently, some complications occurred in our series, especially in our early cases when surgeons were less experienced.

In our series, 8 (0.65%) patients suffered injury at various levels of the biliary tree (3 in the cystic duct, 2 in the CBD and 3 in the intrahepatic ducts). The frequency of this complication is 0%-0.8% in laparoscopic chol ecystectomy[10,12-14], whereas it is very rare in the open procedure. The relatively high incidence of bile duct injury

in laparoscopic cholecystectomy is perceived by many surgeons as the main drawback of the technique. However, as surgeons obtain more experience, the frequency of this complication should decrease.

Bleeding is one of the most frequent and dangerous complications of laparoscopic cholecystectomy. Clinically significant bleeding occurs in 0.5% of laparoscopic cholecystectomy[15]. In our series, bleeding was observed in 19 (1.55%) patients, but in most cases, it was minor and easily controlled. Only 6 (0.49%) patients had bleeding that necessitated conversion to the open procedure. Factors contributing to operative site bleeding may include inadequate exposure, acute inflammation, portal hypertension, adhesions, coagulopathy and rough technique[16]. This was the case in our series, as 5 of the 6 patients who needed conversion to the open procedure were acute admissions. In these patients, inflammatory reaction in the gallbladder fossa prevented adequate exposure of the cystic artery, and transection of the vessel was the end result. In another patient, bleeding occurred from the trocar port in the abdominal wall. Though not significant in amount, its origin could not be revealed and conversion to open was inevitable. Though bleeding is a potentially catastrophic complication, inherent to the laparoscopic technique, it is also the most preventable one, as it is largely related to operator technique.

Wound infection, usually involving the umbilical cannulation site through which the gallbladder is extracted, occurs in 0.3%-1% of cases[4,5,17,18]. Surgical site infection was seen in 4 (0.32%) of our patients, and were all treated successfully with intravenous antibiotics. Postoperative fever occurred in 27 patients. In all cases, the fever was of respiratory origin and resolved with antibiotics and chest physiotherapy.

The average hospital stay was 2.29 d. Recent studies have demonstrated that laparoscopic cholecystectomy can be performed as one day-surgery[19]. In our series, this was true in most uncomplicated, otherwise healthy patients. However, our mean hospital stay was longer, because, when calculating the average length of hospital stay, we included all cases with complications, which required prolonged hospitalization.

In conclusion, in the past few years, much attention has been given to the learning curve of laparoscopic cholecystectomy. Our study adds to and is consistent with the growing body of literature, demonstrating that laparoscopic cholecystectomy is a safe, minimally invasive technique, with favorable results for the patients, provided that the surgeon is meticulous, well-trained and experienced.

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RAPID COMMUNICATION

Value of carcinoembryonic antigen and cytokeratins for the detection of recurrent disease following curative resection of colorectal cancer

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Abstract

AIM: To evaluate the efficacy of postoperative serial assay of carcinoembryonic antigen (CEA) and cytokeratins for the detection of recurrent disease in patients with colorectal adenocarcinoma after radical surgery.

METHODS: Between 1993 and 2000, 120 patients with colorectal adenocarcinoma underwent radical surgery in the Department of Surgical Gastroenterology, Federal University of São Paulo-Escola Paulista de Medicina, São Paulo, Brazil. Periodic postoperative evaluation was performed by assaying markers in peripheral serum, colonoscopy and imaging examination. Presence of CEA was detected using the Delfia® method with 5 μg/L threshold, and cytokeratins using the LIA-mat® TPA-M Prolifigen® method with 72 U/L threshold.

RESULTS: In the first postoperative year, patients without recurrent disease had normal levels of CEA (1.5 ± 0.9 μg/L) and monoclonal tissue polypeptide antigen-M (TPA-M, 64.4 ± 47.8 U/L), while patients with recurrences had high levels of CEA (6.9 ± 9.8 μg/L, $P < 0.01$) and TPA-M (192.2 ± 328.8 U/L, $P < 0.05$). During the second postoperative year, patients without tumor recurrence had normal levels of CEA (2.0 ± 1.8 μg/L) and TPA-M (50.8 ± 38.4 U/L), while patients with recurrence had high levels of CEA (66.3 ± 130.8 μg/L, $P < 0.01$) and TPA-M (442.7 ± 652.8 U/L, $P < 0.05$). The mean follow-up time was 22.3 mo. There was recurrence in 23 cases. Five reoperations were performed without achieving radical excision. Rises in tumor marker levels preceded identification of recurrences: CEA in seven (30%) and TPA-M in eleven individuals (48%).

CONCLUSION: Intensive follow-up by serial assay of CEA and cytokeratins allows early detection of colorectal neoplasm recurrence.

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Key words: Colorectal neoplasms; Cytokeratin; Carcinoembryonic antigen; Residual neoplasm

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INTRODUCTION

Serum tumor markers are substances that can be assayed in peripheral blood. Raised levels of these markers indicate the possible existence of neoplasia in the organism, and thus they form an important instrument in the therapeutic management of such patients[1]. In colorectal adenocarcinoma, carcinoembryonic antigen (CEA) discovered by Gold *et al*[2] in 1965, has been distinguished as a tumor marker with the best characteristics. It is an antigen with high serum levels at the embryonic stage and low levels in adult individuals, and forms the standard for different instances of evaluation[3,4]. Its diagnostic capacity oscillates around 40%[4]. It has a correlation with staging especially among patients with lesions at stage IV[4] of the TNM classification[5] and validity for determining prognostic indices[6]. It can be used as an instrument for determining tumor recurrence[7].

A variety of markers have been developed for better assessing patients with colorectal malignant neoplastic processes, such as CA 19-9[8], CA 242[9], CA 72-4[10], cytokeratins[11], vascular endothelial growth factor (VEGF)[12] and p53[13]. Of these, cytokeratins deserve special attention because of their characteristics. The first method developed for detecting cytokeratins was the tissue polypeptide antigen (TPA) method in 1978[11], evolving into the tissue polypeptide-specific antigen (TPS) method in 1992, using a reagent to identify the M3 epitope of the TPA molecule[14]. Subsequently, in 1994, monoclonal tissue polypeptide antigen (TPA-M) was developed with monoclonal antibodies directed against various epitopes of three different cytokeratins: 8, 18 and 19[15,16]. It has

been studied to evaluate whether it can be utilized for identifying patients with colorectal adenocarcinoma[15,16] and neoplasias in various other organs, such as prostate[17], ovary[18], lung[19], bladder[20] and breast[21].

The objective of the present study was to investigate the value of postoperative serial assay of cytokeratins and CEA, for detecting recurrences of tumors of the colon and rectum, with the aim of determining their possible clinical advantages.

MATERIALS AND METHODS

Patients

Between December 1993 and March 2000, 120 patients with colorectal adenocarcinoma underwent curative surgical treatment in the Department of Surgical Gastroenterology, Federal University of São Paulo-Escola Paulista de Medicina (UNIFESP-EPM), São Paulo, Brazil. Among the patients 69.2% were white, 20.1% brown, 7.7% yellow and 3% black, and 43.2% were male. At the time of diagnosis, their mean age was 62.16 years, ranging from 19 to 89 years. The location of the lesion was the rectum (54.4%), left colon (18.9%), transverse colon (3.6%) and right colon (23.1%). The mean diameter of the tumor was 6.1 cm, ranging from 1 to 17 cm.

The patients were treated according to a protocol approved by the Research Ethics Committee of the institution. The patients were made aware of the study, and signed a statement of consent to their participation in the investigation. Patients with other neoplasias on earlier occasions or those who received previous anti-neoplastic therapy were excluded from the investigation. The operation was considered curative or palliative in accordance with evaluation of the preoperative staging tests, intraoperative evaluation of whether there was residual macroscopic lesion, and verification of the anatomopathological report.

Methods

Staging of the neoplasias was done according to the TNM scheme. Stage I was found in 29, stage II in 21, stage III in 29, stage IV in 41 patients respectively[5]. It was proposed to the patients that evaluations should be made 6, 12, 18, 24, 36, 48 and 60 mo after the operation. Follow-up was performed by clinical assessment, laboratory tests and supplementary examinations such as colonoscopy, computerized tomography (CT) of the abdomen and pelvis, and radiography (X-ray) of the chest using posteroanterior (PA) and lateral views. Opaque enema, nuclear magnetic resonance (NMR) and bone scintigraphy were requested in accordance with the clinical indication in each case.

The CEA and TPA-M markers were evaluated for all patients in the study following the operation, and calculated by patient category (with or without recurrence of neoplasia) for each collection time. The initial collection of blood for assaying the tumor markers was performed while inducing anesthesia prior to the surgical operation. The blood samples were centrifuged with collection of peripheral serum, and stored at -20℃. The serum assays of tumor markers were performed in the Clinical Analysis Laboratory of Hospital São Paulo, UNIFESP-EPM. The CEA concentration was determined using the Delfia® method, taking 5 μg/L as the limit for normalcity[4,7]. Cytokeratins were quantified using the LIA-mat® TPA-M Prolifigen® method, taking 72 U/L as the limit for normality.

Statistical analysis

Statistical analysis was performed using receiver operator characteristic (ROC) curves[22], quantitative analysis of the variables and repeated-measurement variance analysis[23].

RESULTS

In the first postoperative year, patients without tumor recurrence had normal levels of CEA (1.5 ± 0.9 μg/L) and TPA-M (64.4 ± 47.8 U/L), while patients with recurrences had high levels of CEA (6.9 ± 9.8 μg/L, $P < 0.01$) and TPA-M (192.2 ± 328.8 U/L, $P < 0.05$) (Table 1). The serum levels of the tumor markers CEA and TPA-M were measured during the first postoperative year. These parameters could predict recurrences of neoplasia during the second postoperative year. The accuracy was 0.59 (0.43-0.76) for CEA and 0.76 (0.63-0.89) for TPA-M (Figure 1A).

The second postoperative year was the principal period for identifying tumor recurrence. During this year, the CEA levels were normal (2.0 ± 1.8 μg/L) in patients without tumor recurrence, and high (66.3 ± 130.8 μg/L, $P < 0.01$) in patients with recurrences (Table 1), with an accuracy of 0.68 (0.43 - 0.93) (Figure 1B). The circulating TPA-M levels remained normal (50.8 ± 38.4 U/L) in patients without tumor recurrence and high (442.7 ± 652.8 U/L, $P < 0.05$) in patients with recurrences (Table 1), with an accuracy of 0.78 (0.62-0.94) (Figure 1B).

Three (2.5%) out of the 120 patients were lost their follow-up after surgery. The mean time of follow-up was 22.3 mo, ranging 0.2-75 mo. Recurrence of neoplasia was observed in 23 patients (19.2%) during the study period, which was found by clinical, radiological and endoscopic examinations at an average of 18 mo after the initial operation, ranging from 6 to 48 mo. This was preceded by a rise in CEA in 7 patients (30%) and TPA-M in 11 patients (48%).

Among the 23 patients with recurrence of neoplasia, 18 (78.3%) presented multiple tumor recurrences, while 5 (21.7%) who were considered operable underwent surgery for the new lesions. They continued to present residual disease after surgery. Four of these patients died during the postoperative follow-up.

DISCUSSION

One year after the operation, the CEA level was around 1.5 μg/L in the patients without tumor recurrence and around 6.9 μg/L in those with tumor recurrence with an accuracy of 76%. The mean level of TPA-M was around 64.4 U/L in the patients without tumor recurrence and around 192.2 U/L in those with recurrence with an accuracy of 59.1%. In the second postoperative year, the mean CEA level was around 2.0 μg/L in patients without tumor

Table 1 Serum CEA and TPA-M levels by postoperative year (mean ± SD)

	Recurrence of neoplasia	1st yr	2nd yr	3rd yr	4th yr	*P*
CEA (μg/L)	-	1.5 ± 0.9	2.0 ± 1.8	1.4 ± 1.1	1.9 ± 1.0	< 0.01
	+[b]	6.9 ± 9.8	66.3 ± 130.8	19.9 ± 32.0	1.2 ± 0.7	
TPA-M (U/L)	-	64.4 ± 47.8	50.8 ± 38.4	54.0 ± 19.5	43.6 ± 29.0	< 0.05
	+[a]	192.2 ± 328.8	442.7 ± 652.8	93.3 ± 82.0	343.5 ± 437.6	

[a]$P < 0.05$, [b]$P < 0.01$, *vs* no recurrence.

recurrence and around 66.3 μg/L in those with recurrence with a sensitivity of 50% and a specificity of 76.9% and an accuracy of 67.9%. The mean level of circulating TPA-M was around 50.8 U/L in the patients without tumor recurrence and around 442.7 U/L in those with a recurrence, with sensitivity of 75%, a specificity of 71.8% and an accuracy of 78.2%.

Various investigations have suggested that raised level of serum CEA is an important indicator for the recurrence of colorectal neoplasia[4,7]. However, it is not unanimous because of the lack of practical results obtained through clinical follow-up of patients[24]. Nonetheless, periodic postoperative assay of CEA is considered an effective low-cost measure for identifying tumor recurrence[25]. No studies have been undertaken to analyze the efficiency of cytokeratins in detecting recurrences originating from colorectal adenocarcinoma. The present study demonstrated that TPA-M and CEA were comparable in terms of sensitivity, specificity and accuracy in the detection of recurrences. Further studies are needed for evaluating and confirming the importance of TPA-M in following up patients with colon or rectum cancer after surgery.

According to the 2000 consensus of the American Society of Clinical Oncology[25], a postoperative finding of raised CEA concentration in peripheral blood indicates that closer endoscopic and radiological follow-up should be undertaken until such possible tumor recurrences are located. It is certain that patients with colorectal rumor recurrence are never completely cured. Such patients still carry neoplastic cells that are capable of developing when conditions are suitable.

The question is therefore whether intensive follow-up is effective. The medical literature presents conflicting opinions in this respect. One option is to follow up with assay of tumor markers in patients and additional tests if higher than normal concentrations are found[4]. Another alternative is to undergo periodic tests, such as radiological examinations and endoscopic procedures, for rapid identification of reoccurrences of neoplasia and prompt reintervention[6]. Nonetheless, no significant improvements have been achieved in patient survival obtained through close medical follow-up for recurrences of colorectal neoplasia. Minton *et al*[26] showed that reoperation can achieve a five-year disease-free survival. Moertel *et al*[24] carried out interventions in 417 patients with recurrence, and found that 2.3% of them have no relapse in more than one year. Lucha Jr. *et al*[7] achieved a five-year survival in three reoperated patients (1.1%) out of 285 who were

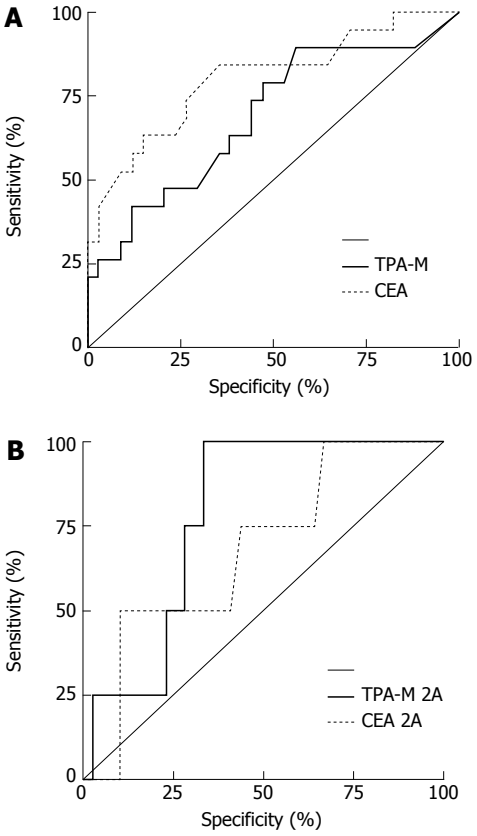


Figure 1 ROC curve for CEA and TPA-M in the first (**A**) and second (**B**) postoperative year among patients with colorectal cancer.

given radical treatment. None of the patients in the present study with recurrence of colorectal neoplasia after the initial resection could be cured. The tumor marker levels are increased in peripheral blood before recurrence of the neoplasia can be identified, which has been confirmed by a recent study[7]. Early surgical intervention at the site of tumor recurrence might become possible through improvements in the imaging methods available[4].

There are some possible explanations why the rates of curative resection of tumor recurrence are low. Sometimes, when resection of a metastasis in a given organ is carried out, the patient may still have micrometastases in other organs, and thus the surgery must be classified as non-radical. Institutional delays cause patients for reintervention with disease that is more advanced than it is when the recurrence is discovered, thereby always obtaining worse results. Moreover, metastases are often multiple, making it difficult to obtain complete resection

and thus compromise the cure for the patient.

Whether intensive follow-up prolongs patient survival is unclear. Ohlsson *et al*[27] evaluated 107 patients in Sweden who were divided into intensive and non-intensive follow-up groups after curative surgery for colorectal neoplasia, and found that there is no statistical difference in the two groups. Secco *et al*[28] followed up 216 patients with colon or rectum cancer undergoing curative surgery for lesion resection (127 of them had intensive follow-up), and found that adherence to a strict medical follow-up program can prolong survival of colorectal cancer patients. There is a consensus that the ideal marker for colorectal neoplasia does not yet exist[29].

Intensive follow-up in the present study enabled early detection of recurrences of colorectal neoplasia, through levels of the tumor markers CEA and TPA-M were increased, which even occurred before the recurrence site was located. Increased CEA and TPA-M levels prior to locating the recurrences occurred in 30% and 48% of the patients with recurrences. However, although reoperation was performed, no cure was obtained.

In conclusion, intensive follow-up by serial assay of CEA and cytokeratins allows early detection of colorectal neoplasm recurrence.

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RAPID COMMUNICATION

Interaction between enteric epithelial cells and Peyer's patch lymphocytes in response to *Shigella* lipopolysaccharide: Effect on nitric oxide and IL-6 release

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Abstract

AIM: To investigate the effect of interaction between enteric epithelial cells and lymphocytes of Peyer's patch on the release of nitric oxide (NO) and IL-6 in response to *Shigella* lipopolysaccharide (LPS).

METHODS: Human colonic epithelial cells (Caco-2) were mixed cocultured with lymphocytes of Peyer's patch from wild-type (C57 mice) and inducible NO synthase knockout mice, and challenged with *Shigella* F2a-12 LPS. Release of NO and mIL-6 was measured by Griess colorimetric assay and enzyme-linked immunosorbent assay (ELISA), respectively.

RESULTS: In the absence of LPS challenge, NO was detected in the culture medium of Caco-2 epithelial cells but not in lymphocytes of Peyer's patch, and the NO release was further up-regulated in both cocultures with lymphocytes from either the wild-type or iNOS knockout mice, with a significantly higher level observed in the coculture with iNOS knockout lymphocytes. After *Shigella* F2a-12 LPS challenge for 24-h, NO production was significantly increased in both Caco-2 alone and the coculture with lymphocytes of Peyer's patch from the wild-type mice but not from iNOS knockout mice. LPS was found to stimulate the release of mIL-6 from lymphocytes, which was suppressed by coculture with Caco-2 epithelial cells. The LPS-induced mIL-6 production in lymphocytes from iNOS knockout mice was significantly greater than that from the wild-type mice.

CONCLUSION: Lymphocytes of Peyer's patch maintain a constitutive basal level of NO production from the enteric epithelial cell Caco-2. LPS-induced mIL-6 release from lymphocytes of Peyer's patch is suppressed by the cocultured epithelial cells. While no changes are detectable in NO production in lymphocytes from both wild-type and iNOS knockout mice before and after LPS challenge, NO from lymphocytes appears to play an inhibitory role in epithelial NO release and their own mIL-6 release in response to LPS.

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Key words: *Shigella* F2a-12 LPS; Colon epithelial cells (Caco-2); Peyer's patch lymphocyte; Coculture; Nitric oxide; Interleukin-6

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INTRODUCTION

The intestinal mucosa is an important route of entry for microbial pathogens. The single layer of enteric epithelial cells that lines the intestinal mucosa is the initial site of interaction between the host and entero-invasive microbial pathogens. Following bacterial entry, intestinal epithelial cells rapidly initiate the innate and acquired immune response. Within the first few hours after bacterial invasion, the intestinal mucosa produces mediators that orchestrate the onset of an early inflammatory response. Characteristics of this program include the increased production and release of chemokine[1], cytokines[2,3] and nitric oxide (NO)[4]. These molecules can act as early signals to activate an acute mucosal inflammatory response and enhance the ability of epithelial cells to produce cytokines that regulate mucosal immune responses.

NO is generated by the conversion of L-arginine to L-citrulline by NO synthase (NOS), which exists in three

isoforms, encoded by a separate gene. The expression of inducible NOS (iNOS, encoded by NOS2, one of the isoforms) is regulated in various cell types and can be upregulated by stimulation with several cytokines or with bacterial lipopolysaccharide (LPS). iNOS can also be expressed constitutively, in some specialized cells, such as human lung epithelial cells[5]. In human colon epithelial cell lines, the iNOS expression and production of NO are upregulated following stimulation with a combination of interferon-γ (IFN-γ) and interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α)[6]. NO can mediate a wide array of physiological effects in the intestine that are relevant to neuronal regulation of vascular function, and epithelial barrier integrity[7]. Moreover, NO produced by intestinal epithelial cells could play a protective role in the host response to enteric microbial pathogens[8,9]. Entero-invasive bacteria directly activate expression of iNOS and NO production in intestinal epithelial cells[10]. The NO in the intestine has been implicated in the suppression of microbial entry into epithelial cells by increasing the protective barrier through the release of epithelial mucus[11] and the induction of intestinal fluid secretion[12]. In addition to direct innate immune action, NO has immuno-regulatory effects relevant to the control of infection on modulating the subsequent acquired immune response[13].

Interleukin 6 (IL-6) is a pleiotropic cytokine that is produced by many different cell types and mediates several physiological responses, such as humoral immune response, acute-phase reactions, and hematopoiesis. In the intestine, IL-6 enhances IgA production in murine Peyer's patch B cells[14] and human appendix B cells that express the IL-6 receptor[15]. Intestinal epithelial cells may also secrete IL-6 and TGF-β to enhance IgA secretion and suppress IgM production by mucosal B cells, whose function is highly dependent upon local production of T cell cytokines[16,17]. These facts indicate that IL-6 plays an important role in the mucosal immune response.

We have previously established a coculture system of human colonic epithelial cells (Caco-2) with murine lymphocytes of Peyer's patch and demonstrated that the cell-cell contact between epithelial and immune cells is important in modulating the epithelial barrier and transport function in response to challenge of *Shigella* LPS[18]. Although accumulating evidence suggests that the interaction or "cross-talk" between epithelial cells and lymphocytes of the intestine is crucial in the immune response to bacterial invasion, no report has clearly demonstrated how the interaction between epithelial cells and lymphocytes, particularly from the Peyer's patch, affects the release of important immuno-regulatory mediators, such as NO and IL-6, at rest and in response to bacterial infection. The present study was undertaken using the established coculture system of Caco-2 epithelial cells with lymphocytes of Peyer's patch to investigate NO and IL-6 release in response to *Shigella* LPS challenge. We also cocultured Caco-2 epithelial cells with lymphocytes from iNOS knockout mice to investigate the involvement of NO in regulation of IL-6 release in response to *Shigella* LPS.

MATERIALS AND METHODS

Isolation of Peyer's patch lymphocytes

Wild-type (C57) mice and iNOS knockout mice of C57 background (SPF, 6-8 wk-old) were obtained from the Animal House of Chinese University of Hong Kong. The lymphoid follicles of the mouse Peyer's patch were excised from the intestinal serosal side and placed in 10 mL PBS, supplemented with 20 mL/L FBS (Invitrogen Co., Grand Island, NY) and 2% penicillin-streptomycin (Invitrogen Co.). The collected patches were triturated by pipetting up and down a few times and smashing through a metallic grid (mesh: 100). Individual lymphocytes were released in the medium below the metallic grid. The lymphocytes were washed with PBS, in which the distribution of Peyer's patch T and B cell populations was consistent with previous data when they were checked by flow cytometry[19], and diluted to a concentration of 1×10^7 cells/mL prior to mixed culture with Caco-2.

Enteric epithelial cell culture

Human colonic cell line Caco-2 was purchased from American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco modified Eagle's minimal essential medium (DMEM; Invitrogen Co.) with 100 mL/L FBS, 2 mmol/L L-glutamine (Invitrogen Co.), 100 μmL/L non-essential amino acid (Invitrogen Co.), 200 units/mL penicillin and 200 μg/ml streptomycin, at 37℃, 50 mL/L CO_2. To maintain the growth of an uniform polarized epithelial monolayer, Caco-2 cells were seeded at a density of 3×10^5 cells on a floating permeable support, which was made of a membrane filter (Millipore, 0.45 μm pore size) with a silicone rubber ring attached on top of it for confining the cells (0.45 cm^2 growth area).

Coculture configurations

Three types of culture configurations were established in this study: (1) Caco-2 culture alone: Caco-2 cells were cultured as a homogenous polarized monolayer according to the epithelial cell culture method mentioned above and they served as the epithelial cell control; (2) Coculture: Caco-2 cells (3×10^5) completely mixed with Peyer's patch lymphocytes (1×10^6), and then were seeded on a permeable support described above and maintained up to the 5th d at 37℃ in a 50 mL/L CO_2 atmosphere; (3) Lymphocytes of Peyer's patch alone: lymphocytes were cultured in an equal volume of the coculture (1×10^6/well in 96-well plate) at 37℃ in a 50 mL/L CO_2 atmosphere as the lymphocyte control.

Shigella F2a-12 LPS pretreatment

When the cells cultured on the membrane filter reached confluence on the 4th d, *Shigella F2a-12* LPS (5 μg/mL, obtained from the Immunology Department of Institute of Microbiology & Epidemiology, Academy of Military Medical Sciences) was added on the apical side of the epithelium monolayer growing on the membrane filters and treated for 24 h at 37℃ in a 50 mL/L CO_2 atmosphere.

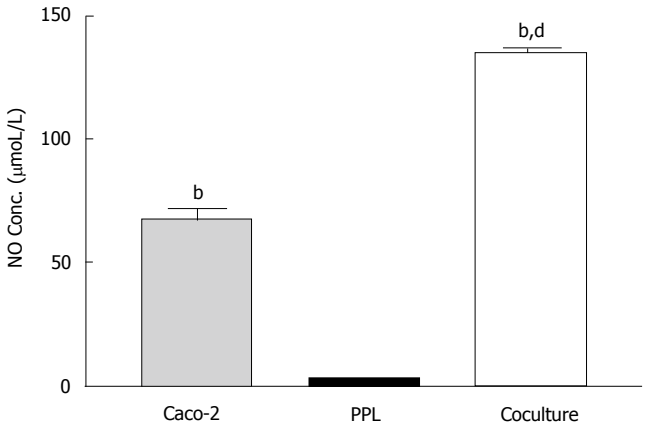


Figure 1 Enhancement of the constitutive NO release in the Caco-2 cocultured with lymphocytes of Peyer's patch. The level of NO in different culture conditions (Caco-2 alone: gray bar; PPL alone: black bar; coculture: white bar) was measured by Griess reagent system and the bar values were derived based on a standard curve. The values represent the means ± SE for 6-12 different samples; $^{b}P < 0.001$ *vs* PPL; $^{d}P < 0.001$ *vs* Caco-2.

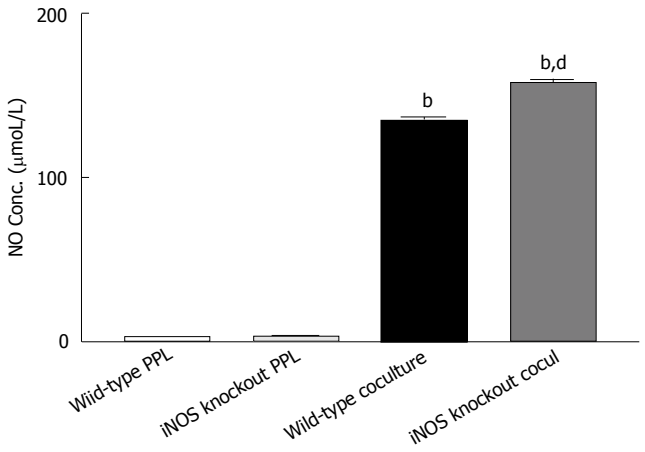


Figure 2 Release of NO is upregulated to the epithelial source Caco-2 cocultured with PPL from iNOS knockout mice. Two sets of cocultures were established (Caco-2 cocultured with PPL of wild-type mice; and Caco-2 cocultured with PPL of iNOS knockout mice). The data are indicated by the following bars (white bar: PPL only from wild-type control mice; light gray bar: PPL only from iNOS knockout mice; black bar: coculture of Caco-2 and PPL from wild-type control mice; dark gray bar: coculture of Caco-2 and PPL from iNOS knockout mice). The level of NO production was measured by Griess colorimetric assay and the bar values were derived based on a standard curve. The values present the means ± SE for 6-8 different samples; $^{b}P < 0.001$ *vs* its own PPL; $^{d}P < 0.001$ *vs* wild-type coculture.

Detection of NO release (Griess colorimetric assay)

After confluent Caco-2 monolayers on the Millipore filter were challenged by *Shigella F2a-12* LPS pretreatment for 24 h, cell culture supernatants were collected and kept at -20℃ until evaluation of NO level in the medium by Griess reagent system (Promega, USA). Briefly, nitrite accumulation was determined by mixing equal volumes of cell culture medium and the Griess reagent (10 mL/L sulfanilamide in 50 mL/L phosphoric acid, and 1 mL/L *N*-1-napthylenediamine dihydrochloride in water). The absorbance (A_{520}-A_{550}) was read on a 96-well microplate reader. Standard curves were constructed with known concentrations of sodium nitrite. The values for the amount of NO were converted from the absorbance values based on the standard curve constructed for each assay.

Enzyme-linked immunosorbent assay (ELISA)

After confluent Caco-2 monolayers on the Millipore filter were challenged by *Shigella F2a-12* LPS pretreatment for 24 h, the culture supernatants were collected and kept at -20℃ until evaluation of mIL-6 level in the culture medium. The IL-6 was measured using a mouse IL-6 quantitative colorimetric sandwich ELISA from Biosource (USA) according to the manufacturer's instructions. The values were calculated on the basis of a standard curve constructed for each assay.

Statistical analysis

All data are expressed as means ± SE. The number of experiments represents independent measurements on separate monolayers. Statistical analysis was performed by repeated measure analysis of variance (ANOVA), and "*P*" values less than 0.05 were considered statistically significant.

RESULTS

Constitutive release of NO from Caco-2 epithelial cells was potentiated by the cocultured lymphocytes of Peyer's patch

In the absence of LPS stimulation, measurements of NO from the culture medium showed a moderate level of NO release from Caco-2 cells (67.64 ± 13.79 μmoL/L) but almost negligible amount (3.15 ± 0.46 μmol/L) in lymphocytes of Peyer's patch from wild-type mice; however coculture of both produced over 2-fold ($P < 0.001$) increase in NO release (Figure 1).

The increase in NO production in the coculture could be due to either potentiation of epithelial release by the cocultured lymphocytes or induction of NO release from lymphocytes by coculturing with epithelial cells. To distinguish this, lymphocytes from iNOS knockout mice were used. If coculture induced NO production in lymphocytes, this would not be observed with iNOS knockout lymphocytes. However, as shown in Figure 2, high level of NO (158.29 ± 2.62 μmoL/L) was still observed in the coculture of Caco-2 with iNOS knockout lymphocytes, confirming the epithelial origin of NO. Moreover, coculure with iNOS knockout lymphocytes produced a level of NO significantly greater than that produced by coculture with lymphocytes of intact iNOS ($P < 0.001$), indicating an inhibitory role of iNOS from lymphocytes in regulating NO release from epithelial cells.

Shigella LPS induced NO release from Caco-2 epithelial cells but with little effect on either lymphocytes of Peyer's patch or the coculture with lymphocytes of iNOS knockout mice

As shown in Figure 3, the level of NO in the culture me-

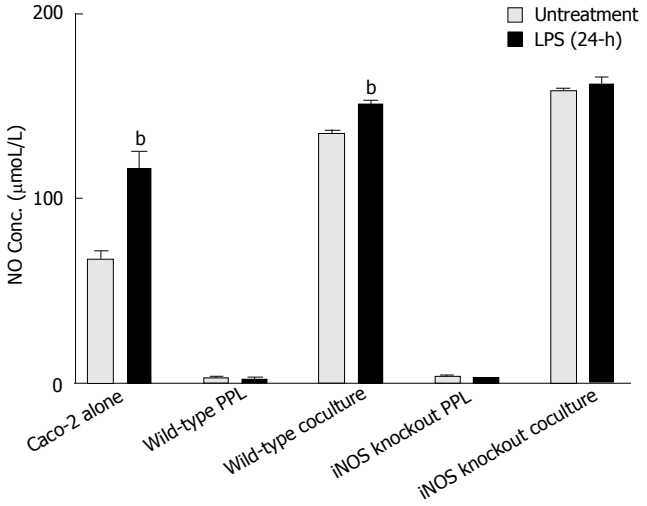


Figure 3 Comparison of NO release induced by *Shigella* LPS in various Caco-2 and PPL culture configurations. *Shigella* LPS was added to five different cultures (set 1: Caco-2 alone; set 2: wild-type PPL only; set 3: Caco-2 and wild-type PPL coculture; set 4: iNOS knockout PPL alone; set 5: Caco-2 and iNOS knockout PPL coculture) and the level of NO release was measured by Griess colorimetric assay and derived based on a standard curve. The black bar and the gray bar represent 24 h treatment with and without LPS, respectively. The values represent the means ± SE for 6-14 different samples. Statistical significance relative to its own un-treatment group was indicated by $^{b}P < 0.001$.

dium of Caco-2 alone and the coculture with lymphocytes of wild-type mice was significantly increased 24 h after LPS treatment as compared to its untreated control (from 67.64 ± 13.80 μmol/L to 116.44 ± 34.57 μmol/L, $P < 0.001$; from 135 ± 4.4 μmol/L to 151.35 ± 4.84 μmoL/L, $P < 0.001$); however, no changes in NO levels were observed in the lymphocytes after LPS treatment (from 3.15 ± 0.46 μmol/L to 2.46 ± 0.52 μmol/L, $P > 0.05$). LPS treatment for 24 h did not significantly produce further increase in the already-high levels of NO release from the coculture of epithelial cells with lymphocytes from iNOS knockout mice (Figure 3).

Suppression of mIL-6 release from lymphocytes of Peyer's patch by cocultured epithelial cells

The release of mIL-6 from different cultures was measured using a mouse ELISA kit. The results showed that mIL-6 release was detected from the lymphocytes of Peyer's patch alone (55.23 ± 2.98 pg/mL) but not from Caco-2 cells, indicating no cross-reactivity of the mouse antibody with human IL-6. When the lymphocytes were cocultured with Caco-2 cells, mIL-6 release was significantly reduced (Figure 4).

LPS triggered mIL-6 release from both lymphocytes of Peyer's patch and cocultures, which was further enhanced with iNOS knockout lymphocytes

As shown in Figure 5, mIL-6 levels in either lymphocytes alone or in cocultured with Caco-2 cells were significantly increased 24 h after *Shigella* F2a-12 LPS treatment. Furthermore, the levels of LPS-induced increases in mIL-6 release were significantly higher in groups with lymphocytes from iNOS knockout mice (Figure 5), indicating an inhibitory role of iNOS from lymphocytes in regulating mIL-6 release.

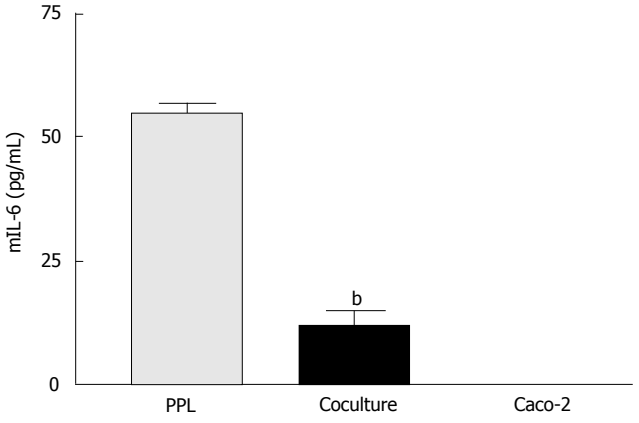


Figure 4 Suppression of mIL-6 release from PPL by cocultured Caco-2 epithelial cells. The bars (gray: wild-type PPL only; black: coculture of Caco-2 and PPL; white: Caco-2 only control) indicate the level of mIL-6 release 4 d after culture. The level of mIL-6 was measured by mIL-6 ELISA and the values were derived based on a standard curve. The values represent means ± SE; $n = 4$; $^{b}P < 0.001$ *vs* PPL.

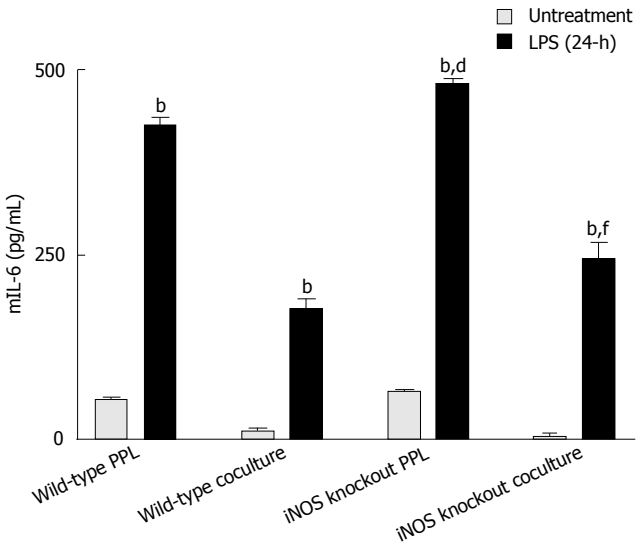


Figure 5 Comparison of mIL-6 release induced by *Shigella* LPS in various Caco-2 and PPL culture configurations. *Shigella* LPS was added to four different cultures (set 1: wild-type PPL only; set 2: Caco-2 and wild-type PPL coculture; set 3: iNOS knockout PPL only; set 4: Caco-2 and iNOS knockout PPL coculture) and the level of mIL-6 release was measured by mIL-6 ELISA and the values were derived based on a standard curve. The black bar and the gray bar represent 24 h treatment with and without LPS, respectively. The values represent the mean ± SE for 4 different samples; $^{b}P < 0.001$ *vs* its own untreatment group, $^{d}P < 0.001$ *vs* wild-type PPL 24-h after challenge *Shigella* LPS and $^{f}P < 0.001$ *vs* wild-type coculture 24-h after *Shigella* LPS challenge.

DISCUSSION

Kerneis S and colleagues[19] have demonstrated that murine Peyer's patch lymphocytes cocultured with human differentiated Caco-2 cell monolayers are able to induce a phenotypic conversion of enterocytes into cells sharing structural and functional properties with M cells. This phenomenon reveals the profound influence of Peyer's patch lymphocytes on intestinal epithelial phenotypes[20,21]. And our previous study indicated that Peyer's patch lymphocytes may modulate intestinal epithelial barrier and ion transport

function in homeostasis and host defense via cell-cell contact and cytokine signaling[18]. The present study has further demonstrated the importance of the interaction between intestinal epithelial cells and lymphocytes of Peyer's patch in regulating the release of NO and IL-6 from Caco-2 epithelial cells and lymphocytes of Peyer's patch, respectively, at rest and in response to *Shigella* LPS challenge.

The present study has demonstrated constitutive release of NO from enteric epithelial cells but not from lymphocytes of Peyer's patch. We also firstly found that the cocultured Peyer's patch lymphocytes potentiated NO release from the enteric epithelial cells. It is known that the constitutive and iNOS activity has the highest level in intestinal villus cells, which is resembled by Caco-2 cells, and the lowest is in crypt cells[22]. Further studies have also demonstrated that the enterocytes regulate their own ion transport processes, either in basal condition or in the presence of active secretion, through the activation of a constitutive NOS-NO pathway, functioning as a braking force of cAMP-induced ion secretion[23]. It has also been reported that iNOS-derived NO is a key mediator of early villous re-epithelialization following acute mucosal injury[24]. These facts indicate that constitutive NO production may have a physiological role in intestinal epithelial cells. It has been proposed that NO synthesis in intestinal epithelial cells is up-regulated through some molecules or cytokines including T-lymphocytes derived cytokines[25] during cell-cell contact with lymphocytes[26]. The observed potentiation of NO release from epithelial cells by Peyer's patch lymphocytes is consistent with the above notion. Our data also suggest that NO from the lymphocytes, although almost negligible, may be involved in negatively regulating NO release from enteric epithelial cells since lymphocytes from iNOS knockout mice further enhance NO release from the epithelial cells. This also rules out the possibility of coculture-induced NO release from the lymphocytes, confirming epithelial release of NO potentiated by the lymphocytes.

Upon *Shigella* F2a-12 LPS challenge, the present results showed that NO from epithelial cell Caco-2 significantly increased but not from lymphocytes of Peyer's patch alone groups. Witthoft[10] has indicated that entero-invasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. NO is involved in the protective mechanisms in the gastrointestinal tract. Enterocyte-derived NO changes intestinal monolayer permeability and ion channel *via* iNOS and COX-2[27-30]. In the acute phase response, *shigellosis* inflammation is characterized by increased cell turnover in the lamina propria (LP) and the epithelium, increased iNOS expression in the surface epithelium, and apoptosis, which seems to be associated with LP macrophages[31]. However, NO production in the coculture with C57 wild type lymphocytes further up-regulated by the LPS treatment, yet there was almost no increase in NO production in the coculture with iNOS knockout lymphocytes following LPS challenge. This observation suggests that the coculture of Caco2 and iNOS knockout lymphocytes has become insensitive to the LPS challenge. The iNOS knockout lymphocytes might lack the additional factors that are crucial for LPS-induced NO release. This is a likely scenario since recent studies demonstrated that host defense function of NO in intestinal mucosal immune response is regulated by IL-12 and/or IFN-γ[32], which are deficient in the iNOS knockout mice[33].

Another interesting finding from the present study is that epithelial cells could also suppress mIL-6 secretion by lymphocytes of Peyer's patch with cell-cell contact. The mIL-6 level in both coculture groups was significantly reduced as compared to that of lymphocytes of Peyer's patch alone. It is clear that IL-6 is produced by a number of cell types including antigen-presenting cells, lymphocytes and even epithelial cells *via* stimulating of endotoxin and a number of other stimuli (TGF-β, IL-1β, etc). However, the mIL-6 in the coculture system is only detectable from mouse lymphocytes of Peyer's patch in our previous study[18]. It should be noted that there is evidence that epithelial cells could inhibit IL-6 secretion from lymphocytes by the interaction between epithelial cells and lymphocytes of Peyer's patch in homeostasis. After *shigella* LPS treatment for 24 h, mIL-6 level was obviously increased in all groups. More interestingly, the LPS-induced mIL-6 release was significantly greater in groups with lymphocytes from iNOS knockout mice, indicating an inhibitory role of iNOS in regulating mIL-6 release from lymphocytes their own. Mucosal production of IL-6 is important because this cytokine may regulate a number of local and systemic immune responses, including IgA secretion[16], macrophage differentiation, T cell proliferation, and acute phage protein synthesis[34] in liver and intestine as well as active transcription of a variety of cytokines and receptors[35]. Accumulating evidence[36] suggests that NO inhibits the LPS-induced IL-6 production and this effect may be a direct effect or caused by inhibited PGE2 production, or both PGE2 and NO may act in an autocrine or in a paracrine manner. NO from epithelial cells is easy to reconcile NF-γB activity with the needs of host defense through autocrine pathway to produce a rapid response to a pathogenic stimulus that is shut down promptly so as to minimize host damage[37]. This study only investigated NO release and mIL-6 secretion from different sources to illustrate the cross-interaction between epithelial cells and lymphocytes in homeostasis and host defense. Further studies are required to identify their regulatory mechanism of NO from intestinal epithelial cells to reduce LPS-induced IL-6 production of Peyer's patch lymphocytes.

In summary, the cross-interaction between intestinal epithelial cells and lymphocytes of Peyer's patch regulates host defense mechanism. Lymphocytes of Peyer's patch can induce a basal constitutive production of NO from epithelial cells. This basal level NO may play an important physiological role in the enteric epithelium in the absence of infection. During infection, NO release from epithelial cells is further upregulated. Conversely, epithelial cells can downregulate IL-6 secretion of Peyer's patch lymphocytes, while LPS can upregulate IL-6 release. Furthermore, NO from lymphocytes appears to play an inhibitory role in the epithelial NO release and their own IL-6 release in response to LPS. Taken together, NO and IL-6 are important immuno-regulatory mediators in response to bacterial infection through the interaction between epithelial cells and Peyer's patch lymphocytes.

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RAPID COMMUNICATION

Deficiency of Adenomatous Polyposis Coli protein in sporadic colorectal adenomas and its associations with clinical phenotype and histology

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Abstract

AIM: To evaluate the frequency of the loss of the Adenomatous Polyposis Coli (APC) protein and to compare the APC status with the characteristics of colorectal adenomas.

METHODS: Immunohistochemical analysis of the APC protein was performed on 118 adenomas and the results were compared with parameters of malignant potential, location of adenomas, macroscopic appearance and age of the patients.

RESULTS: A complete loss of the APC protein was found in 28 (24%) adenomas, while 90 (76%) were APC positive. The mean size of adenomas was 13.5 ± 14.2 mm (95% CI 10.5-16.5) in APC-positive, and 13.8 ± 15.5 mm (95% CI 7.8-19.8) in APC-negative adenomas (P = 0.364). Statistical analysis revealed no difference between APC-positive and negative adenomas as to the histological type (P = 0.327) and grade of dysplasia (P =0.494). We found that even advanced adenomas did not differ in their APC status from the non-advanced tumors (P = 0.414). Finally, no difference was found when the location (P = 0.157), macroscopic appearance (P = 0.571) and age of patients (P = 0.438) were analysed and compared between both APC positive and negative adenomas.

CONCLUSION: Most adenomas expressed full-length APC protein, suggesting that protein expression is not a reliable marker for assessment of *APC* gene mutation. Complete loss of APC protein did not influence morphology, location, or appearance of adenomas, nor was it affected by the patient's age.

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Key words: Adenomatous Polyposis Coli protein; Adenoma; Immunohistochemistry; Histology

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INTRODUCTION

Adenomatous polyposis coli (APC) gene is thought to play a key role in the pathogenesis of colorectal cancer not only in patients with familial adenomatous polyposis syndrome (FAP), but also in the majority of patients with sporadic colorectal neoplasia[1]. About 60%-80% of sporadic carcinomas of the large bowel carry at least one mutation in the *APC* gene and a similar frequency was reported in colorectal adenomas[2-4]. Hence, a mutation in the *APC* gene is suspected to be an early event in the process of colorectal carcinogenesis[5].

There are numerous known, functionally important mutations in the *APC* gene. Almost all of the mutations implicated in the process of the neoplastic conversion lead to a stop codon with subsequent synthesis of a truncated APC protein, which lacks the carboxyl-terminal part[5,6]. Loss of this part of the APC protein results in the absence of almost all binding sites for β-catenin, which, if not bound in a multiprotein complex with APC, translocates into the nucleus, where it promotes the process of malignant transformation[6]. The absence of the full-length and functional APC protein can be detected by immunohistochemistry using the rabbit or mouse polyclonal antibodies[7,8]. Iwamoto *et al* reported the results of an immunohistochemical analysis, where the frequency of the complete loss of the APC protein was substantially lower

in colorectal adenomas than that detected in carcinomas[9]. Another authors also studied the relationship between the APC protein expression and morphological features of colorectal neoplasms, but results are still conflicting[10-12]. Therefore, in our study we investigated the relationship between the loss of the full-length APC protein assessed by immunohistochemistry and some characteristics of colorectal adenomas with special attention to the malignant potential of adenomas.

MATERIALS AND METHODS

Subjects

One hundred and eighteen consecutive patients with endoscopically-removed colorectal adenomas were involved in this study. Patients with known diagnosis of the familial adenomatous polyposis were excluded. In patients with more than one adenoma, only the largest one was used for further assessment. The size of each adenoma was measured, either using the biopsy forceps, having their tips separated in close proximity to the adenoma within the bowel, or after removal of the polyp from the colon. In large, piecemeal, resected tumors, the size was assessed before the adenoma was removed from the bowel. Adenomas arising in the cecum, ascending colon, or transverse colon were defined as right-sided tumors, whereas those arising distally to the splenic flexure were labeled as left-sided adenomas. Appearance of adenomas was defined either as pedunculated, when a visible stalk was present, or sessile, in the absence of a stalk.

All adenomas were evaluated histologically after staining with hematoxylin-eosin by an experienced pathologist (I.V.). An immunohistochemical examination of the APC protein was then performed on all adenomas and evaluated for size, histology and grade of dysplasia, as parameters of the malignant potential. We also included the adenoma's location, its macroscopic appearance and the patient's age in the statistical analysis. An informed consent was obtained from all patients and the study was approved by the Ethics Committee of the General Teaching Hospital, Prague.

Immunohistochemistry of the APC protein

In general, APC immunohistochemistry was performed using the anti-APC antibodies and their dilution as described by Iwamoto *et al*[9,13]. Paraffin-embedded tissue blocks were cut to obtain sections 4 μm thick, deparaffinized in xylen and rehydrated in a series of graded alcohol + water solutions. Endogenous peroxidase activity was then blocked using a 3% solution of hydrogen peroxide in methanol. Subsequently, tissues were incubated in non-immune serum for 30 min. Next, samples were incubated with anti-APC antibodies (C-terminus, Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:200 for 60 min. Streptavidin peroxidase (detection kit) with diaminobenzidine (DAB) as a substrate was used for detection at room temperature. Sections were stained with hematoxylin, dehydrated and assembled into the xylen-soluble medium. A definite APC immune reaction in the adjacent normal mucosa of each adenoma served as a control of the normal APC protein reaction.

Table 1 Demographics of patients and characteristics of adenomas

Patients	*n*		118
Adenomas	Sex	Male/Female	71 (60%)/47 (40%)
	Age (yr)	Mean (range), Median	63 (25-86), 65
	Size (mm)	Mean (range), Median	14 (3-55), 6
	Location	Left/Right	83 (70%)/35 (30%)
	Histology	Tubular/ Tubulo-villous/ Villous	82 (70%)/32 (27%)/4 (3%)
	Dysplasia	Low/High-grade	103 (87%)/15 (13%)
	Appearance	Pedunculated/ Sessile	31 (26%)/87 (74%)

Statistical analysis

Normality was tested by Shapiro-Wilk's W test, as the data did not show a normal distribution, non-parametrical statistical methods were used. The APC status was compared with characteristics of adenomas using the Fisher´s exact test and χ^2 (Chi-square) test for categorical variables and the Mann-Whitney test for continuous variables. All tests were bilateral and the significance level was set at 5% for each analysis. We used the statistical software Statistica, version 7, StatSoft, Inc. (2004).

RESULTS

Patients' demographics and the adenomas' characteristics are shown in Table 1.

Results of immunohistochemistry

Of 118 adenomas evaluated, 90 (76%) expressed full-length APC protein, whereas 28 (24%) were APC protein-negative (Figures 1 and 2). APC status was then compared with the parameters defininig the malignant potential of the adenoma (size, histology and grade of dysplasia) (Table 2).

Comparison of the APC status and characteristics of adenomas

The mean size of APC-positive and negative adenomas was 13.5 mm (SD ± 14.2; 95% CI 10.5-16.5) and 13.8 mm (SD ± 15.5; 95% CI 7.8-19.8), respectively. Using the Mann-Whitney test, no statistically significant difference was found between the two groups ($P = 0.364$). Histological assessment revealed that among 82 tubular adenomas, 20 (24%) were APC-negative, and 62 (76%) were positive for APC by immunostaining. Similarly, of 32 tubulovillous adenomas, eight (25%) were negative, while 24 (75%) were positive for the APC protein. All four villous adenomas expressed the APC protein. No statistically significant relationship was found between the histology and the APC status of adenomas (χ^2 test, $P = 0.327$). Majority of adenomas harboured low-grade dysplasia ($n = 103$). Twenty five (24%) of them were APC-negative, while the remaining 78 (76%) were positive. Three (20%) adenomas with high-grade dysplasia did not show APC positivity, whereas

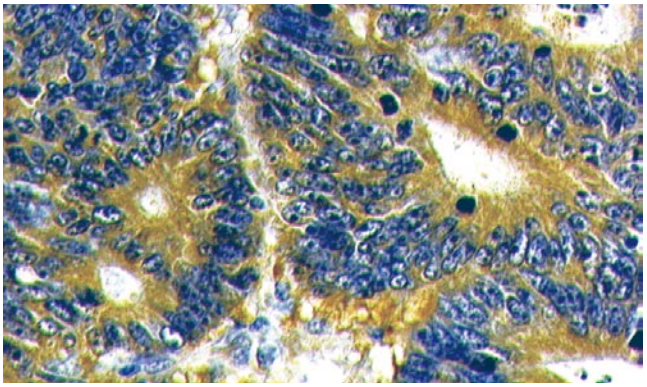


Figure 1 APC immunohistochemistry in colonic adenoma. Difuse positivity (brown color) in the cytoplasma of epithelial cells of the adenoma – presence of the full-lenghth APC protein. Counterstained with hematoxylin. Magnification × 20.

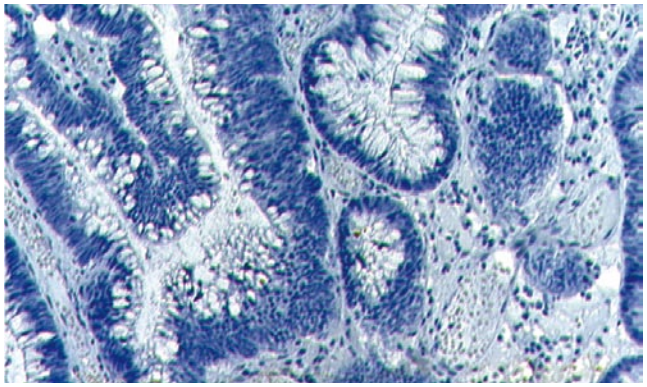


Figure 2 Absence of the immunoreactivity in the cytoplasma of epithelial cells of the adenoma due to the complete loss of the full-lenghth APC protein. Counterstained with hematoxylin. Magnification × 10.

12 (80%) were positive. Again, no significant relationship between the APC presence/absence and the grade of dysplasia was observed in our group (Fisher`s exact test, P = 0.494).

We identified a substantial number of advanced adenomas (n = 46, 39% of entire group). Of them, ten (22%) were APC-negative. Similar results were obtained among non-advanced adenomas, where 18 out of 72 (25%) did not express the APC protein. Using the Fisher's exact test, no significant difference was found between the APC groups (P = 0.414).

Further assessment was performed to look for a relationship between the APC status and the location of adenomas, their macroscopic appearance and the patient's age (Table 2). More than 2/3 of all adenomas were located distally to the splenic flexure. Seventeen left-sided adenomas (20%) were APC-negative, compared with 11 (31%) APC-negative right-sided tumors (Fisher`s exact test, $P$ = 0.157). Comparison between the APC status and the macroscopic appearance of the adenomas revealed 21 (24%) APC-negative, sessile adenomas. Similarly, seven (23%) APC-negative, pedunculated polyps were observed (Fisher`s exact test, P = 0.571). To evaluate the relationship between the age and APC immunoreactivity, patients were divided into groups according to their age: < 41, 41-50, 51-60, and > 60 years of age. Corresponding numbers of APC-negative adenomas were 3 (37%), 4 (36%), 5 (16%), and 16 (24%), respectively. No significant difference was found among the groups (χ^2 test, P = 0.438).

Table 2 Comparison of characteristics of adenomas with APC

Parameter		APC+	APC-	P
Size (SD)		13.5 (± 14.2)	13.8 (±15.5)	0.364[1]
Histology	Tubular	62 (76%)	20 (24%)	
	Tubulovillous	24 (75%)	8 (25%)	0.327[2]
	Villous	4 (100%)	0 (0%)	
Dysplasia	Low-grade	78 (76%)	25 (24%)	0.494[3]
	High-grade	12 (80%)	3 (20%)	
Advancement	Advanced	36 (78%)	10 (22%)	0.414[3]
	Non-advanced	54 (75%)	18 (25%)	
Location	Left	66 (80%)	17 (20%)	0.157[3]
	Right	24 (69%)	11 (31%)	
Appearance	Sessile	66 (76%)	21 (24%)	0.571[3]
	Pedunculated	24(77%)	7 (23%)	
Age (yr)	< 41	5 (63%)	3 (37%)	
	41-50	7 (64%)	4 (36%)	0.438[2]
	51-60	27 (84%)	5 (16%)	
	> 60	51 (76%)	16 (24%)	

[1] Mann-Whitney test; [2] χ^2 (Chi-square) test; [3] Fisher's exact test.

DISCUSSION

We attempted to verify the hypothesis that the absence of the full-length APC protein resulting from a bi-allelic mutation of the *APC* gene (or a mutation in one allele and the loss of the other) might be associated with a higher degree of malignant potential or with an advanced type of adenoma. We found a complete loss of the APC immunoreactivity in a relatively small proportion of adenomas (24%), whereas more than 3/4 of them displayed either complete, or partial positive results in APC immunostaining. This result is, to some degree, inconsistent with the hypothesis that complete inactivation of both alleles of the APC gene occurs early during the process of colorectal tumorigenesis. These data were derived from studies demonstrating a similar frequency of *APC* mutation, about 60%-80%, in both carcinomas and adenomas[2,3-5]. Contrary to this, our results might indicate that the large number of adenomas contain the mutation of only one allele of the *APC* gene. Whereas 45%-60% of all sporadic adenomas and 60%-80% of carcinomas harbour one inactivated allele, a bi-allelic defect could be found in approximately 30% of adenomas[5,8]. One possible explanation for this finding is that the carcinogenesis of the sporadic colorectal tumors differs from that in patients with familial adenomatous polyposis syndrome, where the vast majority of adenomas harbour a bi-allelic mutation of the *APC* gene[14,15].

However, it is necessary to emphasize that immu-

nohistochemistry detects the presence of the full-length APC protein instead of the actual mutation of the gene. It is the current assumption that almost all of the *APC* gene's mutations result in the production of a truncated protein that has lost its tumor suppressor function[6]. The absence of the full-length APC protein thus indicates a bi-allelic loss of the gene, caused by two mutations or by the combination of one allele's mutation with another type of genetic or epigenetic event (allelic loss or promoter hypermethylation). No clear relationship between the APC protein expression and adenomas' morphology was observed in our study. While some authors found higher frequency of the *APC* gene mutations in tubulovillous and villous adenomas[16], others refuted this observation[9]. The lack of significant difference between adenomas with different malignant potential is contrasted by an abrupt increase in the APC protein loss in carcinomas, which harbour a complete (bi-allelic) *APC* defect much more frequently. Such results were published by Iwamoto *et al*, who found completely negative response for APC immunostaining in 83% of carcinomas, but in only 29% of adenomas[7]. Moreover, even when the exact proportion of *APC* gene mutations or other epigenetic changes were assessed, results were different for adenomas compared with carcinomas[5,8]. On the other hand, Chen *et al* demonstrated positivity of the APC protein detected immunohistochemically and by western blot analysis in the majority of colon cancers[17]. In this study, the APC protein levels were not different among tissues with unchanged or mutated *APC* gene. Even hypermethylation of the *APC* gene, which seems to be a relatively frequent mechanism of APC silencing[18], did not result in APC protein negativity.

Sporadic adenomas may grow with only one allele inactivated[19] as a result of, for example, dominant-negative effect of APC protein homodimers formed by a truncated protein together with the full-length APC protein[20]. These homodimers prevent normal function of APC protein but not its immunoreactivity. This theory is supported by the finding of the nuclear, so that abnormal accumulation of β-catenin in adenomas with a definite positive immunohistochemical result of the APC protein[7]. Therefore, the presence of the APC immunostaining does not necessarily mean the presence of functioning APC protein. Furthermore, mutations of APC may have direct oncogenic effect. Tighe *et al* suggested that the initial APC mutation acts as a "double whammy", destabilising the genome and setting the stage for deregulated proliferation upon loss of the second APC allele[21].

A key question is whether the presence, or absence, of the full-length APC protein affects the prognosis of adenomas as to their future malignant transformation. Theoretically, the lack of the APC protein should stimulate adenomas to grow and undergo malignant transformation. While some reports, including our results, showed no different morphology of APC-negative and positive adenomas[7,9], others found that the prevalence of *APC* mutation continuously increased in the transformation from normal mucosa to carcinoma[8], or that the frequency of *APC* mutations in tubulovillous or villous adenomas was higher than in tubular adenomas[10]. Among our adenoma specimens, a lack of APC protein was observed even in some polyps smaller than 5 mm, which are generally thought to be of minimal clinical importance. On the other hand, several investigators have observed that there are many other genes involved in colorectal carcinogenesis and that a significant number of adenomas, as well as carcinomas, do not harbor any *APC* gene mutations at all[8,16,22]. It should also be noted that colorectal cancers are genetically heterogeneous, multiple genes and mutation may be involved in the tumorigenesis simultaneously as well as the key mechanism may be different in different parts of the colon.

In conclusion, we have not observed a relationship between the presence, or absence, of the full-length APC protein and histology as well as clinical phenotype of sporadic colorectal adenomas. Moreover, only a small part of adenomas did not express the APC protein as assessed by immunohistochemistry. Both these results may indicate that adenomas either harbour genetically heterogenous background, or that one allele remains unchanged and still allows production of normal, full-length APC protein. Hence, immunohistochemistry seems to be an unreliable marker for assessment of *APC* gene changes in colorectal adenomas.

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RAPID COMMUNICATION

How much helpful is the capsule endoscopy for the diagnosis of small bowel lesions?

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Abstract

AIM: To assess the practically usefulness and diagnostic yield of this new method in a group of patients with suspected small bowel lesions.

METHODS: Capsule endoscopic (CE) examination by using M2A capsule endoscope TM (Given Imaging, Yoqneam, Israel) was performed in thirty nine patients (26 males, 13 females) with suspected small intestinal lesions. The composing of the patients was as follows: obscure gastrointestinal bleeding in twenty three patients, known Crohn's disease in 6 patients, in whom CE was used to evaluate the severity and extension of the diseases, chronic diarrhea in 8 patients, abdominal pain in one patient and malignancy in one patient with unknown origin.

RESULTS: In two patients CE failed. Different abnormalities were revealed in 26 patients overall. Detection rate of abnormalities was highest among patients with obscure gastrointestinal bleeding and the source of bleeding was demonstrated in 17 of 23 patients with obscure bleeding (73.9%). Entero-Behcet was diagnosed in two patients by CE as a source of obscure gastrointestinal bleeding. In 6 patients with known Crohn's disease, CE revealed better evaluation of the disease extension. In 3 of 8 (37.5%) patients with chronic diarrhea; CE revealed some mucosal abnormalities as the cause of chronic diarrhea. In a patient with unexplained abdominal pain and in a cancer patient with unknown origin, CE examination was normal.

CONCLUSION: In our relatively small series, we found that capsule endoscopy is a useful diagnostic tool particularly in diagnosis of obscure gastrointestinal bleeding, chronic diarrhea and in estimating the extension of Crohn's disease.

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INTRODUCTION

Demonstration of small bowel lesions was always difficult due to its inaccessibility in conventional modalities. Currently available diagnostic methods such as enteroclysis or push enteroscopy are far from satisfactory results regarding diagnostic yields, patients comfort and technical ease. The technology used in present study permits us to demonstrate the whole small bowel, which was relatively not possible by using conventional modalities. When the diseased area is located to the small bowel, endoscopic evaluation can be extremely difficult. Push enteroscopy has the ability to demonstrate only the proximal to mid-jejunal lesions[1,2]. Endoscopic imaging of the entire small bowel can only be carried out by sonde enteroscopy or surgically assisted enteroscopy which are invasive and uncomfortable techniques[3-6]. On the other hand, capsule endoscopy (CE) allows painless, non-invasive and to get physiologic imaging of the small bowel lesions. Many studies have demonstrated that this technique is superior to the conventional investigations.

In this study, our aim was to investigate the diagnostic capability of CE in different small bowel pathologies in which conventional modalities failed to demonstrate the organic lesions.

MATERIALS AND METHODS

All patients undergoing CE were from February, 2002 to February, 2005 at the Department of Gastroenterology of Hacettepe Medical Center. Thirty nine patients (26 males, 13 females) were evaluated (Table 1). The mean age of the patients was 43 ± 17 years (range, 13-83 years). The indications for the study were obscure gastrointestinal bleeding (23 patients), evaluation of Crohn's disease (6 patients), chronic diarrhea (8 patients), chronic abdominal

Table 1 Indications and findings of capsule endoscopy in thirty nine patients

No	Age (yr)	Sex (F/M)	Indication	Diagnosis/Finding	Comp
1	83	M	OGB	Angiodysplasia in ascending colon	(-)
2	50	M	OGB	Angiodysplasia in distal intestine (with Billroth I gastrectomy for ulcer)	(-)
3	74	M	OGB	Normal	(-)
4	29	M	Diarrhea	Normal	(-)
5	54	M	Diarrhea	Inadequate recording time	(-)
6	56	M	Cancer with unknown origin	Normal	(-)
7	35	M	OGB	Ulcer in distal intestine (CD)	(-)
8	62	M	OGB	The capsule failed to pass the pyloric channel	(-)
9	59	M	OGB	Vegetan mass (Jejunal metastases of operated renal cell carcinoma)	(-)
10	29	M	OGB	Ulcers in distal intestine (CD)	(-)
11	46	M	OGB	Ulcers in proximal intestine (Vasculitis)	(-)
12	58	F	Diarrhea	Ulcers in proximal intestine (with Billroth II gastrectomy for gastric lymphoma)	(-)
13	16	M	OGB	Normal	(-)
14	52	F	OGB	Normal	(-)
15	59	F	OGB	Normal	(-)
16	23	M	CD	Ulcers in distal intestine	(-)
17	70	M	OGB	Angiodysplasia in proximal intestine and ulcers in distal intestine (Behcet's Disease)	(-)
18	39	M	OGB	Ulcer in proximal intestine (GIST in operation)	(-)
19	73	F	OGB	Active bleeding in proximal intestine	(-)
20	54	F	OGB	Aphthous ulcers in distal intestine (CD)	(-)
21	18	M	CD	Ulcers and pseudopolyps in distal intestine	(-)
22	63	M	OGB	Multiple angiodysplasia in distal and proximal intestine	(-)
23	22	M	Diarrhea	Polyp-like lesions (BLH) and Hymenolepsis Nana in all small intestine	(-)
24	20	M	Diarrhea	Normal	(-)
25	71	F	OGB	Normal (with Billroth I gastrectomy for ulcer)	(-)
26	44	F	Diarrhea	Normal	(-)
27	32	M	CD	Deep ulcers in distal intestine	(-)
28	43	M	OGB	Active bleeding in proximal intestine (Angiodysplasia in operation)	(-)
29	21	M	Diarrhea	Polyp-like lesion in proximal intestine (BLH)	(-)
30	42	F	OGB	Active bleeding in proximal intestine (Angiodysplasia in operation)	(-)
31	34	F	Diarrhea	Normal	(-)
32	60	M	OGB	Angiodysplasia	(-)
33	13	F	OGB	Angiodysplasia in proximal intestine	(-)
34	39	M	CD	Ulcers in distal intestine	(-)
35	41	M	OGB	Punched ulcers in distal intestine (Behcet's Disease)	(-)
36	38	F	AP	Normal	(-)
37	27	F	CD	Small ulcers in small intestine	(-)
38	48	M	CD	Ulcer and hyperemia in terminal ileum	(-)
39	52	F	OGB	Angiodysplasia in proximal intestine	(-)

OGB: Obscure gastrointestinal bleeding, CD: Crohn's Disease, AP: Abdominal pain, BLH: Benign lymphoid hyperplasia.

pain (one patient), and malignancy with unknown origin (one patient) (Table 2). All patients were undergone by upper gastrointestinal endoscopy and colonoscopy, as well as small bowel follow-through barium studies to exclude any structuring lesions. Some patients were undergone by these endoscopic procedures more than two times when bloody mucosa exists during first examination.

Table 2 Indications for capsule endoscopy

Indication of patient for CE	Number of patients
Obscure gastrointestinal bleeding	23
Crohn's disease	6
Chronic diarrhea	8
Abdominal pain	1
Cancer of unknown origin	1
Total patients	39

Technique used

All patients swallowed Given M2A video capsule (Given Imaging Yoqneam, Israel) after a 10-12 h fasting period. Laxative preparations were not used. The sensors were attached to eight locations on the anterior abdominal wall. A belt containing data recorder was positioned outside the anterior abdominal wall. Patients then swallowed the M2A Capsule with a mouthful of water. The patients remained nil orally for 2 h. Then they allowed to drink clear fluid and after an additional 2 h were allowed to resume their daily diet and activity if their conditions were good enough to do so. After completion of the examination, the recorder was removed and data were transferred to a com-

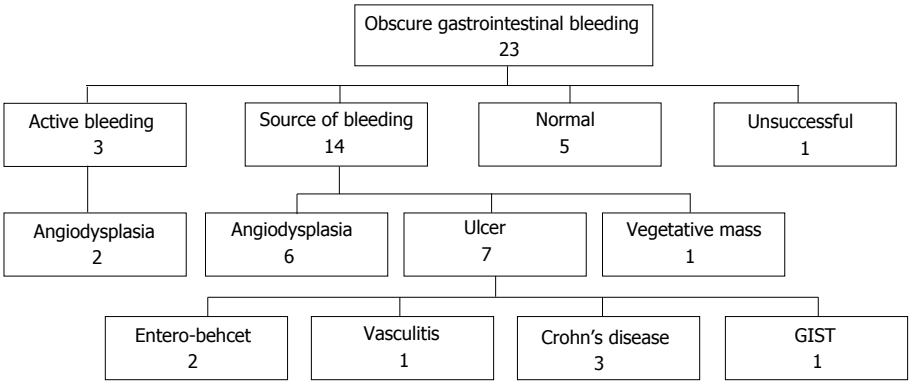


Figure 1 Results of obscure gastrointestinal bleeding.



Figure 2 Angiodysplasia in small intestine without evidence of active bleeding.



Figure 3 Jejunal invasion of recurrent renal cell carcinoma.



Figure 4 Punched-out ulcer in Behcet' s disease.



Figure 5 Capsule endoscopic findings of Crohn's disease in small bowel: large ulcer in ileum.



Figure 6 Polyp-like lesions located entire small intestine: benign lymphoid hyperplasia.

puter workstation through a high capacity digital link. The images were reviewed by 2 gastroenterologists.

RESULTS

CE examinations were unsuccessful in two patients. In one elderly patient with obscure gastrointestinal bleeding, the capsule failed to pass the pyloric channel since the patient was in supine position because of his medical condition. In another patient with chronic diarrhea, CE examination was unsuccessful after recording about 3 h.

Two patients with obscure gastrointestinal bleeding had undergone surgery for complicated peptic ulcer and one patient with chronic diarrhea for gastric lymphoma in the past (Table 1).

The average gastric emptying time (based on 35 patients) was 37 ± 30 min. (range 3 to 113 min.). In 28 of the 39 patients, the capsule passed the ileocecal valve within the duration of the examination. The mean small bowel transit time (based on 26 patients) was 280 ± 101 min. (range 149 to 484 min.). Neither had symptoms of intestinal hurrying, nor were on prokinetic drugs and encountered complain related to the capsule used. Potentially bleeding sites were identified in 17 patients (73.9%) including active bleeding area (three patients), ulcer (seven patients), angiodysplasia (six patients) and vegetative mass (one patient) (Figure 1).

In three of twenty-three patients with obscure gastrointestinal bleeding; active bleeding (fresh blood) sites were noted as in proximal small intestine, but the source of the bleeding was not clearly seen. In two of the three, the bleeding sources were identified as angiodysplasia located at proximal small intestine related to operation. Six patients had angiodysplasia noted (3 proximal intestine, 1 proximal and distal intestine, 1 distal intestine, 1 colon) without evidence of active bleeding (Figure 2). These lesions were thought to be the cause of the bleeding. Colonic angiodysplasia missed during colonoscopy. In one patient, a vegetative mass was demonstrated in the proximal jejunum which was missed during abdominal CT scan small bowel series. This patient was operated and diagnosed as a jejunal invasion of recurrent renal cell carcinoma (Figure 3). Very interestingly, in two patients with obscure gastrointestinal bleeding, CE demonstrated ulcerations and/or angiodysplasia in terminal ileum which are specific lesions for Behcet's disease (Figure 4). In five patients with obscure gastrointestinal bleeding single or multiple ulcers were

found. In the three of five patients, typical ulcers in distal intestine have been identified in which abdominal CT scan small bowel series were negative for typical findings of Crohn's disease. In one patient with vasculitis, CE found multiple ulcers in proximal intestine. Leiomyosarcoma was demonstrated in one of those patients through the histopathologic examination of the surgical specimen. In those five patients neither active bleeding nor a positive source of bleeding was demonstrated.

Of the six patients with known Crohn's disease, all had obvious ileal ulcer (Figure 5).

As seen in Table 1, in 2 of the eight patients with chronic diarrhea typical benign lymphoid nodular hyperplasia was demonstrated on entire small bowel (Figure 6). Many parasites looks-like *Hymenolepsis nana* were demonstrated throughout the small intestine of one patient with benign lymphoid hyperplasia. His feces were examined carefully and cystic forms of giardia and eggs of *Hymenolepsis nana* were demonstrated. In one patient with Billroth II because of stomach lymphoma, CE found multiple ulcers in small intestine.

In a patient with unexplained abdominal pain and in another cancer patient with unknown origin, CE didn't reveal any abnormalities.

DISCUSSION

In daily practice, available imaging techniques of the small intestine consists of push-endoscopy and X-Ray studies such as small bowel series and enteroclysis. In most cases upper endoscopy can easily reach to the second part of the duodenum. Enteroscopy can demonstrate more extent sites of intestine, but maximally only reach to the mid-jejunum[1,2]. Biopsy is also possible during enteroscopy. Push and sonde enteroscopies have been used for revealing the small intestinal lesions, but these techniques are not easy to neither carry out nor give a high diagnostic yield. In radiological studies, diagnostic accuracy of any small bowel pathology is often low as well as being very uncomfortable. Overall visualization of the mid and distal portion of small bowel is unsatisfactory. Regarding the difficulty for evaluation of occult GI bleeding, which has often been attributed to a source in the small intestine, the many patients finally undergo surgery without knowing the actual source of bleeding.

CE has shown a good diagnostic tool in patients with obscure gastrointestinal bleeding[7-14]. In several clinical studies, it has been shown that this modality may be superior to push enteroscopy[15-21] , small bowel series[7,22], enteroclysis[23] and CT scan[24] in identifying small bowel lesions in obscure gastrointestinal bleeding. Currently, obscure GI bleeding is very common[25]. The diagnostic yield of CE for the suspected bleeding source in obscure GI bleeding has been reported up to from 38% to 93%[9]. In this study, this modality demonstrated the source of bleeding in 17 of the 23 patients (73.9%) with obscure GI bleeding. In our two cases diagnosed as Behcet's disease with obscure GI bleeding, CE revealed multiple punched-out ulcers in which some are bleeding located at terminal ileum. The punched-out ulcer was first time demonstrated in the literatures by using CE.

Definitive diagnoses included angiodysplasia 53%, malignancy 6.3% and inflammatory disease 6.3%[15]. In our study the most important finding is that CE clearly demonstrated angiodysplasia in 6 patients (26%).

Intestinal metastases from renal cell carcinoma are rare. The most common clinical presentation of intestinal metastases of renal cell carcinoma is intestinal bleeding[26]. In the literature, one patient with a history of renal-cell carcinoma had a mass in the proximal small bowel found by using CE[27]. In our study, we diagnosed metastatic tumors originating from renal cell carcinoma in one patient who had severe gastrointestinal bleeding and required 25 units blood transfusion. Small bowel follow-through barium series and abdominal CT scan didn't reveal any abnormalities but CE demonstrated the tumor mass and bleeding area. Only in five patients with obscure GI bleeding, CE did not find any abnormalities.

CE is a superior and more sensitive diagnostic tool than barium follow-through and entero-computerised tomography in patients with suspected Crohn's disease[24,28]. CE is effective in diagnosing patients with suspected Crohn's disease undetected by using conventional diagnostic methods[29-33]. In six cases with known Crohn's disease who underwent CE as a part of routine evaluation of the effectiveness of the treatment, we found obvious ileal ulcers that were missed during small bowel series. Additionally, in three patients with obscure gastrointestinal bleeding, there were typical ileal ulcers and pseudopolyps for Crohn's disease. It is clear that in these three patients CE was very helpful for diagnosis of such abnormalities. On the other hand, this modality took the images out is only lasting 2 to 4 h.

CE did not play an important role in the evaluation of patients with chronic abdominal pain of unknown origin[34]. In patients with undiagnosed abdominal pain the yield of CE appears to be low[35]. In our patients with unexplained abdominal pain CE did not find any abnormalities.

In conclusion, this modality is an exiting new diagnostic technique allowing us to visualize an important section of the gut, which previously has been considered an inaccessible area. When compared with push enteroscopy and small bowel radiography, CE is superior in diagnostic accuracy. In particular for Crohn's disease, CE was is very useful for making the diagnosis and determining the extent and severity of involvement.

In our relatively small series, we found that CE is a useful endoscopic technique particularly in diagnosing obscure gastrointestinal bleeding, chronic diarrhea and Crohn's disease.

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RAPID COMMUNICATION

Circulating DNA level is negatively associated with the long-term survival of hepatocellular carcinoma patients

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Abstract

AIM: To quantify the circulating DNA in plasma from patients with hepatocellular carcinoma (HCC) and to evaluate its prognostic value.

METHODS: Blood samples were collected from 79 patients with HCC before operation, 20 patients with liver cirrhosis, and 20 healthy volunteers. Circulating DNA was extracted from plasma and quantified. The association between circulating DNA level and prognosis of HCC patients was evaluated.

RESULTS: Compared with the healthy volunteers (17.6 ± 9.5 ng/mL), a significant higher circulating DNA level was found in the patients with HCC (47.1 ± 43.7 ng/mL, P = 0.000) or with liver cirrhosis (30.0 ± 13.3 ng/mL, P = 0.002). The circulating DNA level was closely associated with tumor size (P = 0.008) and TNM stage (P = 0.040), negatively associated with the 3-year disease-free survival (DFS) (P = 0.017) and overall survival (OS) (P = 0.001).

CONCLUSION: Large or invasive tumor may release more circulating DNA, and higher level of circulating DNA may be associated with poor prognosis of HCC patients.

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Key words: Hepatocellular carcinoma; Prognosis; Circulating DNA

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignancies worldwide, and has been ranked the 2nd cancer killer in China since 1990s. The age-standardized mortality rate in China is as high as 34.7/100 000, accounting for 53% of all liver cancer deaths worldwide[1]. Although encouraging long-term survival of HCC patients has been obtained in some clinical centers, recurrence after surgical resection remains one of the major obstacles to further prolonging survival of HCC patients and the overall dismal outcome of patients with HCC has not been completely changed[2,3]. The extremely poor prognosis of HCC is largely due to a high rate of recurrence after surgery or intra-hepatic metastases that develop through invasion of the portal vein or spread to other parts of the liver[4,5].

Identifying biomarker with prognostic significance, especially for predicting recurrence has become an important aspect in HCC study[6]. Since patients with cancers, particularly with high metastatic potential, tend to have higher levels of DNA in plasma, and the molecular biologic findings of circulating DNA in cancer patients are mimicked to those of cancer cells, plasma DNA may be a candidate because of its less invasiveness, simple manipulation, and prognostic information available before operation[7,8]. Few reports have shown the existence of circulating DNA in HCC patients[9-11]. However, the association between circulating DNA level and prognosis of HCC patients is still unclear. In this study, we quantified the circulating DNA in plasma from patients with HCC and evaluated its prognostic value.

MATERIALS AND METHODS

Patients and their clinicopathological characteristics

Seventy-nine patients who received surgical treatment for HCC at Liver Cancer Institute (Zhongshan Hospital) of Fudan University during August-December 2001 were enrolled in this study. The mean age of the patients was 51 years (range 21-83 years). All the patients had a normal liver function preoperatively. All tumors were histopathologically diagnosed as HCC (Table 1). TNM stages of the patients were classified according to the UICC TNM classification of primary liver cancer (6th

edition)[12]. All the 79 patients were given informed consent and followed up till January 2005.

Sample collection

Peripheral blood samples (5 mL) were collected from the HCC patients before surgical operation and put into an EDTA tube. Blood samples from 20 patients with compensated liver cirrhosis (histopathologically diagnosed by biopsy) and 20 healthy volunteers were collected as control. The blood samples were centrifuged at 3000 r/min for 10 min to separate buff coats and plasma. An additional centrifugation for 10 min was performed to produce cell-free plasma. Blood samples were then stored at -80℃ prior to DNA extraction.

Plasma DNA quantification

Plasma and control lymphocyte DNAs were extracted with QIAamp® DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the blood and body fluid protocol of the manufacturer. Two milliliters of plasma was used, a DNA elution volume of 300 μL was obtained after extraction, and 10 μL of plasma DNA was mixed with equivalence 1:3000 SYBR green I (fluorescence dye). The mixture was analyzed by ultraviolet transilluminator system and photographed under stimulating wave of 345 nm and absorbing wave of 500 nm. The diagram was analyzed by software system to read its intensity[13]. The quantity of plasma DNA was calculated by regression equation which was established by intensity of standard DNA content.

Statistical analysis

Results were expressed as mean ± SD. Fisher's exact test was used to compare qualitative data. $P < 0.05$ was considered statistically significant. Continuous variables were transformed into dichotomous variables before survival analysis. Student's *t*-test was used for mean comparisons. Patients' survival was calculated from the data of operation to the data of death or to the data of point. Survival curves were estimated according to the method of Kaplan-Meier and compared with a log-rank test. To identify the factors that might be of independent significance in influencing the disease-free survival (DFS) or overall survival (OS), a Cox stepwise proportional risk regression model was fitted. All statistical analyses were performed with Stata (version 7) statistical software.

RESULTS

Compared with the healthy volunteers (17.6 ± 9.5 ng/mL), a significant higher circulating plasma DNA level was found in the patients with HCC (47.1 ± 43.7 ng/mL, $P = 0.000$) or with liver cirrhosis (30.0 ± 13.3 ng/mL, $P = 0.002$). However, no significant difference was found in the circulating plasma DNA levels between the patients with HCC and liver cirrhosis ($P = 0.191$).

When we used 36.6ng/mL (mean healthy volunteers +2*SD)[14] as cutoff point of circulating DNA level, the 79 HCC patients were divided into high plasma DNA level group (HDNA) (≥ 36.6 ng/mL) and low plasma DNA level group (LDNA) (< 36.6 ng/mL). Significant differences were found in tumor size ($P = 0.008$) and TNM stage ($P = 0.040$) between the two groups of HCC patients (Table 1).

Table 1 Circulating DNA level and clinicopathological features of HCC patients

Variable	*n*	Circulating DNA level		
		HDNA	LDNA	*P*
Tumor number				0.26
Single	60	29	31	
Multiple	19	12	7	
Tumor size				0.008
≤ 5 cm	44	17	27	
> 5 cm	35	24	11	
Tumor capsule				0.9
(+)	41	21	20	
(-)	38	20	18	
Differentiation				0.823
I-II	24	12	12	
III-IV	55	29	26	
Vascular invasion				0.179
(+)	31	19	12	
(-)	48	22	26	
Serum AFP				0.79
≤ 20 μg/L	24	13	11	
> 20 μg/L	55	28	27	
HBsAg				0.266
(+)	67	33	34	
(-)	12	8	4	
Cirrhosis				0.401
(+)	68	34	34	
(-)	11	7	4	
TNM stage				0.04
I-II	49	21	28	
III-IV	30	20	10	
3-yr DFS				0.008
Yes	27	9	18	
No	52	32	20	
3-yr OS				0
Yes	33	10	23	
No	46	31	15	

HDNA: High plasma DNA level (≥ 36.6 ng/mL); LDNA: Low plasma DNA level (< 36.6 ng/mL); DFS: Disease-free survival; OS: Overall survival.

The 3-year DFS rates for HDNA group and LDNA group were 22% and 47% respectively ($P = 0.008$), while the 3-year OS rates were 24% and 61% respectively ($P = 0.000$) (Table 1). High circulating DNA level was independently associated with a poorer DFS and OS ($P = 0.004$, $P = 0.000$) (Figure 1A and Figure 1B).

DISCUSSION

The first discovery of extracellular nucleic acids in the circulation was reported in 1948 by Mandel and Métais[15], who demonstrated the presence of both DNA and RNA in the plasma of healthy and sick individuals. This work was particularly remarkable as it was reported only 4 years after the demonstration of DNA as the material of inheritance[16], and it even preceded the landmark paper by Watson and Crick[17] on the double helical structure of DNA. However, their work did not attract much attention, and further development of the field had to wait some

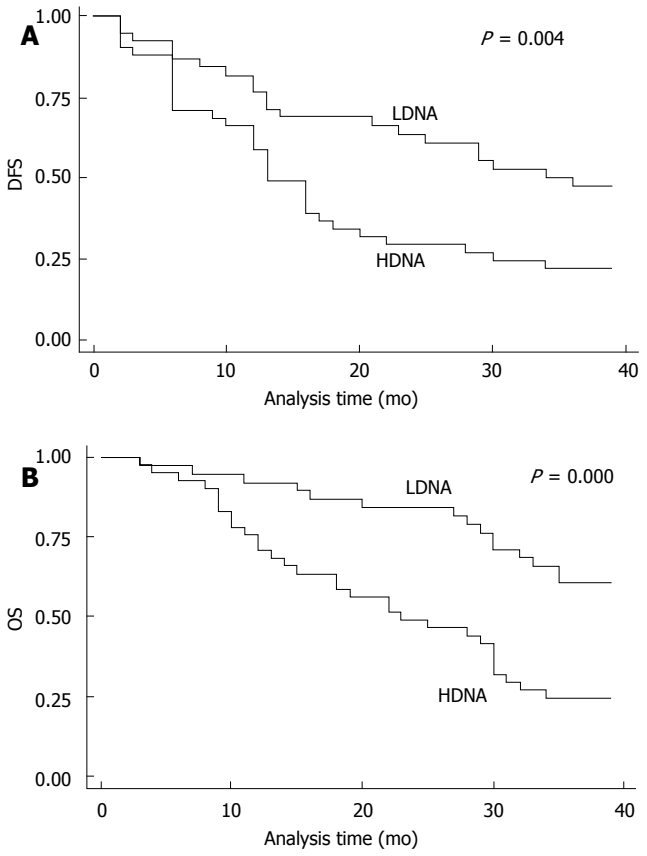


Figure 1 Disease-free (**A**) and overall survival (**B**) curves for HCC patients evaluated according to circulating DNA level. HDNA: High plasma DNA level (⩾ 36.6 ng/mL); LDNA: Low plasma DNA level (< 36.6 ng/mL); DFS: Disease-free survival; OS: Overall survival.

30 years until Leon[18] showed that cancer patients have much higher concentration of circulating DNA than those with non-malignant disease, which is decreased in some cases after anti-cancer therapy. Since then, several follow-up studies have been done to establish the possible value of free circulating DNA as a prognostic factor in patients with pancreatic carcinoma[19], esophageal adenocarcinoma[20], lung cancer[21], melanoma[22] and nasopharyngeal carcinoma[23].

Both plasma and serum samples have frequently been used in the analysis of cell-free DNA. Of particular interest is that the concentrations in serum samples are significantly higher than those in matched plasma samples, which is mainly generated *in vitro* by lysis of white blood cells, suggesting that serum is not suitable for monitoring the concentration of cell-free DNA[24]. A recent work performed a quantitative comparison of matched serum and plasma DNA in patients with colorectal liver metastases and found that serum and plasma DNA level is not correlated with colorectal liver metastases. Furthermore, while serum DNA is significantly associated with the presence of metastases, only plasma DNA is predictive of recurrence, indicating that serum DNA might represent an indirect but tumor-related process, and that plasma DNA better reflects the *in vivo* levels of circulating DNA[14]. Different blood processing protocol may also influence the level of circulating DNA[25]. The plasma obtained by centrifugation alone at various speeds, without subsequent microcentrifugation, has been shown to have substantial amounts of cellular components which may led to the detection of aberrantly high total concentrations of plasma DNA[26]. Therefore, in our study, an additional centrifuge for 10 min was performed to produce cell-free plasma. In order to exclude interference of cell death caused by hepatitis or liver resection, normal liver function test is required for patients' blood sample collection before operation.

In our study, according to the cutoff point of circulating DNA level, the 79 HCC patients were divided into high plasma DNA level group (HDNA) and low plasma DNA level group (LDNA). In HDNA group, 59% (24 of 41) patients had tumor size larger than 5cm while there were only 29% (11 of 38) patients had tumor size larger than 5cm in LDNA group ($P = 0.008$). We also observed that there were 49% (20 of 41) patients with TNM stage III/IV in HDNA group, while there were only 26% (10 of 38) patients with TNM stage III/IV in LDNA group ($P = 0.040$). The mean circulating plasma DNA level was significantly associated with tumor size and TNM stage, indicating that large or invasive tumor may release more circulating DNA and higher level of plasma circulating DNA may be associated with poor prognosis.

Multivariate analysis further confirmed that high circulating DNA level was an independent predictor for poorer 3-year DFS and OS. Furthermore, among patients with TNM stage III or IV, there was no remarkable difference in the 3-year DFS and 3-year OS between HDNA group and LDNA group ($P = 1.000$, $P = 0.448$), while among patients with TNM stage I or II, the 3-year DFS and 3-year OS of patients with HDNA were 24% (5/21) and 38% (8/21), which were much lower than those of patients with LDNA, the 3-year DFS was 57% (16/28) ($P = 0.020$), the 3-year OS was 75% (21/28) ($P = 0.009$). These data suggest that circulating DNA level is negatively associated with the long-term survival of hepatocellular carcinoma patients and might be supplementary to traditional TNM staging.

In conclusion, circulating DNA in liver cancers opens a new research area and eventually be comes a suitable source for development of noninvasive diagnostic methods for HCC.

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RAPID COMMUNICATION

Eradication of *H pylori* infection in a rural population: One-day quadruple therapy versus 7-day triple therapy

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Abstract

AIM: To compare the one-day quadruple therapy with a standard 7-d triple therapy for *H pylori* eradication in a rural population of China.

METHODS: A total of 396 patients with ^{13}C-urea breath test positive for *H pylori* were assigned into two groups: 239 patients received one-day quadruple therapy (amoxicillin 2000 mg qid; metronidazole 500 mg qid; bismuth citrate 900 mg qid and lansoprazole 60 mg once daily) and 157 patients received 7-d standard triple therapy (amoxicillin 1000 mg bid; clarithromycin 500 mg bid and lansoprazole 30 mg bid). All the patients underwent a ^{13}C-UBT to assess the eradication of *H pylori* infection six weeks after treatment.

RESULTS: Two hundred and twenty-nine patients completed the one-day therapy (95.8%) and 148 patients completed the 7-d therapy (94.2%). The one-day therapy eradicated *H pylori* infection in 64 patients (27.95%). In contrast, 103 patients (69.59%) were *H pylori* negative after the 7-d therapy ($P < 0.01$).

CONCLUSION: This pilot study suggests there is no beneficial effect of the one-day therapy in treatment of *H pylori* infection compared with the 7-d standard therapy.

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Key words: Eradication; *H pylori* infection; Quadruple therapy; Triple therapy

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INTRODUCTION

H pylori infection is the most common cause of gastritis, gastric ulcer, duodenal ulcer, and primary gastric MALT-lymphoma, and plays an important role in the development of gastric cancer[1-5].

Linqu County is a rural area with a high risk of gastric cancer in northern part of China, where 72% of adults and 52% of children aged 3-4 years are infected with *H pylori*[6]. Our previous studies carried out in this region suggested that *H pylori* infection is associated with an increased risk of gastric cancer[7,8] and a random trial in Linqu has demonstrated that one-time eradication of *H pylori* infection can yieldcan a significant benefit on precancerous gastric lesions and a favorable trend on gastric cancer development[9].

Multiple therapies against *H pylori* infection are available at present with a higher cure rate than the single-antibiotic regimens. A triple-drug regimen, consisting of a proton-pump inhibitor, clarithromycin and amoxicillin for over a period of 7 d, has been recommended as a first-line therapy in *H pylori* management[10,11].

In 2003, Lara reported an efficacy of 95% with one-day quadruple therapy for the eradication of *H pylori* in comparison with 90% of 7-d triple therapy[12]. The advantages of the one-day therapy are low costs, low antibiotic resistance and a higher compliance, which motivated us to conduct a similar trial in Linqu County, Shandong Province, China. Herein, we report the results of a random trial of one-day quadruple therapy *vs* standard 7-d triple therapy in a rural population at high-risk of gastric cancer in China.

MATERIALS AND METHODS

Study design

This was a prospective, open, standard controlled therapeutic trial. Subjects with positive ^{13}C-urea breath test (^{13}C-UBT) were allocated to receive one-day therapy and 7-d standard therapy respectively. The efficacy of the treatment was assessed six weeks after the therapy.

Clinical characteristics of the groups

A total of 654 healthy residents aged 25-49 years from 3 villages were invited to participate in this study. All participants received a ^{13}C-UBT to determine *H pylori* infection status. Trained field staff from the Peking University School of Oncology interviewed all the participants using a structured questionnaire containing age, sex, address, occupation, and previous histories of peptic ulcers, use of antibiotics and any allergic reaction to antibiotics. Exclusion criteria for the trial included negative *H pylori* infection, age younger than 25 years or older than 49 years, pregnant or breast-feeding women and people with allergic reaction or severe concomitant illness (e.g. cardiac, respiratory, renal, hepatic diseases and bleeding ulcer). Finally, 396 subjects from 3 villages qualified for entering this trial as illustrated in Figure 1. Among them, 239 patients from two villages (Xinzhuang and Zhujiagou) received a one-day quadruple therapy, and 157 patients from another village (Shiyazuei) received a 7-d triple therapy. The project was approved by the Institutional Review Board of the Peking University School of Oncology and an informed written consent was obtained from all the patients.

Assessment of H pylori status

Details of the assay of ^{13}C-UBT were described previously[13,14]. All participants were fasted overnight before baseline samples of exhaled CO_2 were collected the following morning. Each patient was then requested to drink 20 mL of water with a pill of 80 mg ^{13}C-urea (> 99%). Exhaled CO_2 was collected in sampling tubes 30 min later. $^{13}CO_2$ values were determined using a gas isotopic ratio mass spectrometer, and any concentration of $^{13}CO_2$ at 30 min that exceeded the baseline concentration more than 4 parts per 1000 (> 0.4%) was regarded as a positive result. Samples of duplication were tested with an agreement of 97.2%. The sensitivity and specificity of the test were 94% and 83%, respectively[15].

All the ^{13}C-UBT assays were blinded to the treatment aims. The end point of the study was the proportion of the patients with negative results of the ^{13}C-UBT 6 wk after treatment in each aim.

Treatment

In the one-day treatment group, a quadruple regimen consisted of amoxicillin 2000 mg qid; metronidazole 500 mg qid; bismuth citrate 900 mg qid and lansoprazole 60 mg once daily. And in the 7-d treatment group, the triple regimens consisted of amoxicillin 1000 mg bid, clarithromycin 500 mg bid and lansoprazole 30 mg bid.

To maintain a high compliance, all the patients in each village were organized into 4-5 groups and each group took medications in one household. The staff from the Peking University School of Oncology visited each household and monitored the medications for each patient at 8:00 and 20:00 everyday for the 7-d therapy and at 7:00, 13:00, 19:00 and 1:00 for the one-day therapy.

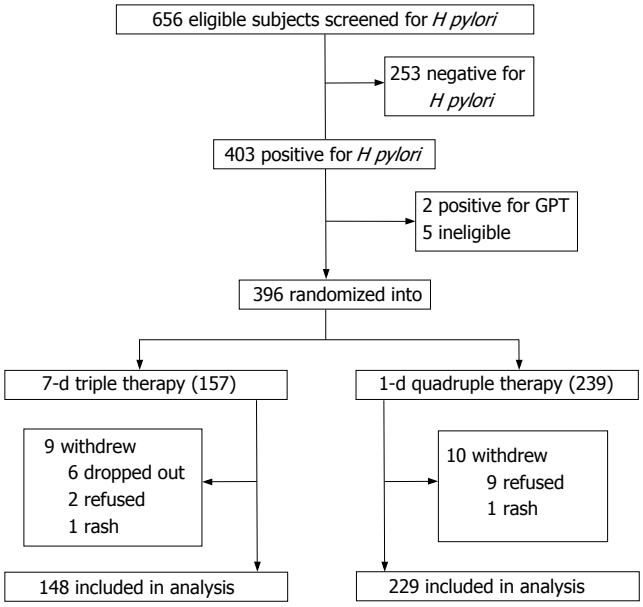


Figure 1 Trial profile.

Table 1 Demographic data and patient compliance in two groups

Treatment	*H pylori* (+)	Age	Completed	Compliance (%)
1-d	239	39.3 ± 6.9	229 (M105, F124)	95.8
7-d	157	39.5 ± 7.3	148 (M78, F70)	94.3
Overall	396	39.4 ± 7.1	377 (M183, F194)	95.2

Monitoring of adverse events

To monitor the adverse reaction related to the anti-*H pylori* therapies, each patient was interviewed using a questionnaire at six days after the treatment. Since metronidazole may injure the hepatic function, 2 mL blood was collected from each fasted patient before, 6 d and 6 wk after the treatment to test glutamic-pyruvic transaminase (GPT) in the laboratory of Linqu County Hospital.

Statistical analysis

For the efficacy analysis, the differences between the two eradication rates were assessed by the chi-square testing. A two-sided 95% confidence interval for the difference between success rates was calculated. All tests were two-sided and values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Two hundred and twenty-nine patients (95.8%) completed the one-day therapy and 148 patients (94.3%) completed the 7-d therapy (Table 1). The treatment was unsuccessful for 10 patients in the one-day therapy group and 9 patients

Table 2 Eradication of *H pylori* in two groups

Treatment	*n*	*H pylori* (-)	Eradication (95% CI)	*P*
1-d	229	64	27.95% (22.13-33.76)	
				< 0.01
7-d	148	103	69.59% (62.18-77.00)	

in the 7-d therapy group. In the one-day therapy group, 9 refused the treatment and 1 had to withdraw because of severe rash. Of the 9 patients in the 7-d therapy group, 2 refused the treatment, 6 dropped out and 1 had to withdraw because of severe rash also.

H pylori infection was eradicated in 64 of 229 patients (27.95%) in the one-day therapy compared with 103 of 148 patients (69.59%) in the 7-d therapy six weeks after the treatment (Table 2).

As it shows in Table 3, the proportions of skin rash (5.4%) and abdominal bloating (12.8%) in the 7-d therapy group were significantly higher than that of the one-day therapy group ($P = 0.013$ and $P = 0.035$). In the one-day therapy group, the proportion of stool discoloration (33.2%) was significantly increased ($P < 0.001$).

All 377 patients had a normal hepatic function before the treatment. Six days after the treatment, 91 patients (24.1%) showed abnormal GPT (> 40 IU), and in 9 patients (2.4%) GPT exceeded 100 IU, including 3 from the one-day therapy group and 6 from the 7-d therapy group. In the one-day therapy group, 26.6% of the patients (61/229) had a temporarily abnormal hepatic function (GPT > 40 IU) compared with 24.3% of the patients (36/148) in the 7-d therapy group. However, 36 d later, GPT level of most patients declined to below 40 IU.

Table 3 Incidence of side effects in each group

	Treatment group			
Side effects	7-d	*n* (%)	1-d	*n*(%)
Total		148		229
Rash		8 (5.4)[1]		2 (0.01)
Diarrhea		12 (8.1)		10 (4.4)
Abdominal pain		3 (2.0)		10 (4.4)
Abdominal bloating		19 (12.8)[1]		15 (6.6)
Stool discoloration		14 (9.5)		76 (33.2)[1]
Headache		5 (3.4)		4 (1.75)
Dizziness		4 (2.7)		8 (3.5)
Nausea		15 (10.1)		17 (7.4)
Metallic taste		0		4 (1.75)
Loss of appetite		5 (3.4)		7 (3.1)
Increase of appetite		14 (9.5)		13 (5.7)
Disappearance of abdominal pain		1 (0.007)		1 (0.004)
Stools becoming normal		8 (5.4)[1]		3 (1.3)
Feeling better		3 (2.0)		17 (7.4)[1]

[1]There was significant difference between the two groups ($P < 0.05$).

DISCUSSION

The rates of *H pylori* eradication were 27.95% by one-day therapy and 69.59% by 7-d therapy in this pilot study. It was similar to one of our previous studies (64%)[9,16], in which 3411 patients received 14-d therapy with amoxicillin 1000 mg bid and omeprazole 20 mg bid. Wong *et al* reported 76.4% of eradication of *H pylori* by 7-d triple therapy in a trial in Changle County, China[17]. The result of the one-day anti-*H pylori* therapy in Linqu does not support Lara's report[12]. The eradication rate of *H pylori* is lower (27.95%) than that reported by Lara (95%).

In our study, quality control was enforced throughout the trial. The patients in the one-day therapy group were confined to the village throughout the medication day and monitored by our staff according to the schedule. Although a few patients were out of the village during the 7 d treatment, they were allowed to take the medicine during away and required to contact our staff by cell phone immediately after medicine was taken on schedule.

The reason for the poor eradication rate of *H pylori* in one-day therapy is unclear. The antibiotics are widely used in China and all over the world. As a result, antibiotic resistance is a growing and severe problem worldwide. Kato *et al* reported the prevalence of *H pylori* resistance to clarithromycin, metronidazole and amoxicillin is 29%, 24% and 0% (51 of *H pylori* isolated strains) among Japanese children[18]. Kim *et al* studied 652 *H pylori* isolated strains from Korea residents, and the resistance rates were 40.6% (metronidazole), 5.9% (clarithromycin), 5.3% (tetracycline), and the resistance to metronidazole increased from 1994 to 1999 (from 33.3 to 47.7%)[19]. Li reported the *H pylori* resistance to metronidazole in Chinese is between 28% and 80%, and to clarithromycin is < 5%, respectively[20]. Although the relatively high-dosages of antibiotics are used in the one-day therapy, it is worth investigating the time frame associated with the effectiveness of proton-pump inhibitors and antibiotics. If the proton-pump inhibitor plays a more effective role in the middle or late stage of the treatment in combination with antibiotics, one-day therapy may result in low efficacy in eradication of *H pylori*. In general, the efficiency of *H pylori* eradication in general population is lower than that in clinical patients. The clinical patients with active inflammation seem to be more sensitive to anti-*H pylori* therapy than those with long-term infection and with severe precancerous lesions.

In conclusion, our trial confirms a high efficacy rate of eradication of *H pylori* infection by a standard 7-d triple therapy; whereas, one-day quadruple therapy with high dosage of amoxicillin, metronidazole, bismuth citrate and lansoprazole produces a low eradication rate of *H pylori*. Because the efficacy of *H pylori* eradication reported by Lara *et al* can not be confirmed, the one-day quadruple therapy could not be recommended to use in a large scale study in future.

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RAPID COMMUNICATION

Significance of serum IgA in patients with acute hepatitis E virus infection

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Abstract

AIM: To study the significance of serum anti-hepatitis E virus (HEV) IgA in patients with hepatitis E.

METHODS: A new method was established to assay anti-HEV IgA, which could be detected in the middle phase of the infection. We compared anti-HEV IgA assay with anti-HEV IgM and anti-HEV IgG assay in sera from 60 patients with positive HEV-RNA.

RESULTS: The 60 patients with positive HEV-RNA had both anti-HEV IgA and anti-HEV IgM and 410 patients with negative HEV-RNA were used as control. Periodic serum samples obtained from 60 patients with hepatitis E were tested for HEV RNA, anti-HEV IgM, anti-HEV IgA and anti-HEV IgG. Their HEV-RNA was detectable in the serum until 20 ± 11 d. We used anti-HEV IgM and anti-HEV IgA assay to detect HEV infection and positive results were found in 90 ± 15 d and 120 ± 23 d respectively, the positive rate of anti-HEV IgA was higher than that of anti-HEV IgM and HEV-RNA (P <0.05).

CONCLUSION: The duration of anti-HEV IgA in serum is longer than that of anti-HEV IgM, and anti-HEV IgA assay is a good method to detect HEV infection.

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Key words: Hepatitis E virus; RNA; Anti-HEV IgM; Anti-HEV IgA

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INTRODUCTION

Hepatitis E is caused by infection with hepatitis E virus (HEV), which is a non-enveloped single-stranded RNA virus. Just like hepatitis A virus (HAV), HEV is usually transmitted from person-to-person via the fecal-oral route. Epidemic of hepatitis E has been found in Central and South-East Asia, North and West Africa, and Mexico, where fecal contamination of drinking water is common. However, recent reports showed that HEV occurs in individuals without traveling in HEV epidemic areas in developed countries like the United States of America and Japan[1-5]. The application of recently developed serologic tests has revealed anti-HEV in almost all countries, including developed countries, in which the disease virtually does not occur[6-8]. Possible reservoirs of HEV in the developed countries may be found in animals like monkeys, pigs, cows, rodents, sheep or goats, suggesting that HEV may be zoonotic[9-12]. Xia *et al*[13] reported that acute hepatitis E occurs in rhesus monkeys, indicating that transfusion transmits hepatitis E. Hepatitis-E is a self-limited disease. But a current research indicates that there are cis-reactive elements in HEV ORF3[14]. Surjit M *et al*[15] found that enhanced alpha microglobulin secretion from Hepatitis E virus ORF3-expressing human hepatoma cells is mediated by the tumor susceptible gene 101. HEV is highly epidemic in China and it is very important for us to find a reliable assay for diagnosing its acute, sub-clinical and chronic infection.

Immunoglobulin M (IgM) against HEV (anti-HEV IgM) is used as a reliable and sensitive marker for recent HEV infection[16-19]. However, the specificity of solid-phase assay for anti-HEV IgM has been questioned in patients with IgM-rheumatoid factors in serum. It was reported that anti-HEV IgA can be utilized as an additional complementary antibody for detecting recent HEV infection[20-23]. But the clinical and epidemiological implications of anti-HEV IgA in finding HEV infection remain to be clarified.

In this study, we used the solid-phase assay to detect HEV-specific IgA. The kinetics of anti-HEV IgM, anti-HEV IgA and anti-HEV IgG responses in serum from patients with hepatitis E demonstrated that anti-HEV IgA assay could identify HEV infection.

MATERIALS AND METHODS

Serum samples

This study enrolled 100 patients (77 males and 23 females,

mean age 52 ± 11 years) with acute hepatitis E admitted to the Department of Infectious Diseases of Tongji Hospital in Wuhan, China, during the period of August 2003 - August 2004. Sera were collected from 60 HEV-RNA positive patients on d 15, 30, 60, 90 and 120 after the onset of symptoms. In addition, 410 serum samples obtained from HEV-RNA negative patients (304 males and 106 females, mean age 42 ± 9 years) were used as controls, including 100 samples from voluntary blood donors with normal alanine aminotransferase (ALT) level, 218 samples from patients with hepatitis A, B or C, 52 samples from patients with autoimmune hepatitis, 40 samples from patients with rheumatoid arthritis. Liver function tests included total serum bilirubin (T-Bil) and direct bilirubin (D-Bil, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total protein, γ-glutamyltranspeptidase, prothrombin time, and total cholesterol levels. The serum samples were stored at -70℃ until HEV RNA test.

The presence of anti-HAV IgM, hepatitis B surface antigen (HbsAg), hepatitis B e antigen (HbeAg), antibodies to HBV core (anti-Hbc), and anti-HCV was determined with commercially available kits.

RNA extraction and RT-PCR

Total RNA was extracted with guanidinium isothiocyanate. RT-PCR was carried out in a 50 μL reaction volume containing 10 μL purified RNA, 25 pmol each primer, 1 μL AMV reverse transcriptase and 0.5 μL Taq polymerase. Thirty-five cycles of PCR amplification were performed at 94℃ for 1 min, at 52℃ for 45 s, at 72℃ for 1 min, and a final extension at 72℃ for 7 min. Second-round reactions were carried out in a volume of 50 μL containing 10 μL first-round product, 25 pmol each primer and 0.5 μL Taq polymerase. Thirty PCR omplification cycles were performed at 94℃ for 1 min, at 52℃ for 45 s, at 72℃ for 1 min, and a final extension at 72℃ for 7 min.

Consensus oligonucleotide primers for ORF2 were designed within regions of conserved sequences in the full-length sequences from Asia, Mexico, USA and China strains. The ORF2 primers, at positions 5681nt-6486nt, produced a product of 805 bp nucleotide: EP1 (5´-GCTTCAAATTATGCCCAGTA-3') and EP2 (5' -TGTTGGTTGTCATAATCCTG-3'). Second round internal primers amplified 345 bp products for ORF2: EP3 (5´-GTTATGCTTTGCATA(T)CATGG-3') and EP4 (5' -CCGACGAAATCAATTCTGTC- 3').

Sequence analysis

Second-round PCR products were purified and ligated into pMD 18-T vectors. Twenty-five clones were sequenced automatically with an ABI model - 3730 DNA sequencer and the ABI sequencing ready reaction kit (Perkin - Elmer) according to the manufacturer's instructions. Sequences used for phylogenetic analysis were obtained from Mexico (GenBank accession no. M74506), Burma (D10330), USA (AF060669), China (C1, AJ272108; C2, AF103940; C3, AF151963).

Phylogenetic analysis

Nucleotide sequences were aligned using Clustalx 1.81 (http://downloads.scalableinformatics.com/downloads/clustalx/). These alignments were analyzed with Mega 2.0 (http://evolution.genetics.washington.edu/phylip/software.html) to evaluate the evolutionary distances between sequences using 1000 bootstraps of the data. Bootstrap values greater than 70% could provide significant evidence for phylogenetic analysis grouping.

ELISA for anti-HEV-IgM and anti-HEV-IgG

Anti-HEV-IgM and anti-HEV-IgG were detected by using commercially available kits (BeiJing Wantai Biological Pharmacy Enterprise Co., LTD.). Absorbance (*A*) value was measured at 450 nm. Anti-HEV-IgM cut off = 0.26 + negative control mean *A* value, Anti-HEV-IgG cut off = 0.16 + negative control mean *A* value. Samples with *A* values > cut off were considered positive, Samples with *A* values ≤ cut off were considered negative.

ELISA for anti-HEV-IgA

ELISA for detection of IgG using purified recombinant ORF2/ORF3 protein (WanTai biological pharmacy enterprise co. LTD.) was performed with HRP-labeled coat monoclonal anti-human IgA (Wuhan Lingfei Science and Technology Limited Company) in stead of HRP-labeled coat monoclonal anti-human IgG. In order to detect the titers of goat anti-human IgA-HRP, ELISA was performed with 6 diluted concentrations: 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000. Results demonstrated that P/N (ratio of sample *A* to negative *A*) of 1:4000-1:8000 of diluted antibodies was ≥ 2.0. In order to obtain the stably detectable results, the diluted concentration of 1:4000 was adopted as standard one and 410 sera with negative HEV-IgM and HEV–IgG were detected for HEV-IgA.

Statistical analysis

Chi square test was used for statistical analysis.

RESULTS

Among the 100 acute phase sera with hepatitis E, 60 (60%) were positive for HEV RNA by reverse transcription- nested polymerase chain reaction (RT- nPCR) using the degenerated primers. Among which 25 partial ORF2 sequences (5972-6317 nt) belonging to HEV genotype Ⅳ were analyzed.

HEV RNA in serum samples from 100 donors negative for anti-HEV-IgM and anti-HEV-IgG was detected by anti-HEV-IgA assay. The absorbance (A) was set at 0.128, standard deviation (SD) at 0.053. When critical value was set at A + 2SD, the IgA assay results were all negative in 410 HEV-IgM and -IgG negative sera. When the A value of positive HEV-IgA assay was set as the mean value of negative control plus 0.18, the specificity of anti-HEV IgA was verified by absorption with the same recombinant ORF2 protein (50 mg/L at the final concentration for anti-HEV IgA assay). Briefly, prior to assay, 10 serum samples were diluted at 1:100, 1:500, 1:1000, 1:5000 to make its A value < 1.5. The microwells were incubated at 37℃ for 0.5 h, then at 4℃ overnight. When the ten serum samples were diluted at 1:500, the A value of the tested samples

Table 1 Prevalence of anti-HEV IgM and anti-HEV IgA in different groups of subjects

Group	*n*	Age (mean ± SD)	Anti-HEV IgA, *n* (%)	Anti-HE IgM, *n* (%)	Anti-HEV IgG, *n* (%)	HEV RNA, *n* (%)
Hepatitis A	62	33.1 ± 11.2	2 (3.2)	1 (1.6)	1 (1.6)	0
Hepatitis B	100	36.3 ± 12.2	1 (1)	0	10 (10)	0
Hepatitis C	56	45.6 ± 15.3	1 (1.8)	1 (1.8)	1 (1.8)	0
Autoimmune hepatitis	52	40.5 ± 10.1	0	2 (3.8)	3 (5.8)	0
Rheumatoid arthritis	40	56.4 ± 9.4	0	2 (5)	1 (2.5)	0
Blood donors	100	36.8 ± 18.5	2 (2)	1 (1)	8 (8)	1 (1)
Hepatitis E	100	52.6 ± 14.8	100 (100)	100 (100)	98 (98)	60 (60)

Table 2 Serum anti-HEV IgA, anti-HEV IgM, HEV RNA, ALT and TBil

T/mo	HEV RNA *n* (%)	Anti-HEV -IgA *n* (%)	Anti-HEV -IgM *n* (%)	Anti-HEV -IgG *n* (%)	ALT (mean ± SD) nkat/L	TBil (mean ± SD) μmol/L
-0.5	60 (100)	60 (100)	60 (100)	59 (98)	1117 ± 200	86 ± 17
-1	36 (60)	60 (100)	60 (100)	60 (100)	4101 ± 884	506 ± 123
-2	3 (5)	58 (97)[a]	46 (77)	60 (100)	1850 ± 750	198 ± 68
-3	0	56 (93)[b]	34 (57)	60 (100)	800 ± 250	84 ± 31
-4	0	38 (3)[b]	12 (20)	60 (100)	567 ± 183	46 ± 14
-5	0	18 (0)[b]	2 (3)	60 (100)	467 ± 150	23 ± 8

[a]$P < 0.05$, [b]$P < 0.01$ compared with anti-HEV IgM positive results.

was reduced by ⩾ 75% in the anti-HEV IgA assay with the recombinant ORF2 protein, the sample was considered positive for anti-HEV.

Among the serum samples obtained from 100 blood donors, only one sample was positive for anti-HEV IgM, and two samples were positive for anti-HEV IgA. The same samples were positive for anti-HEV IgG and HEV RNA. Using the cutoff values, among remaining 310 serum samples obtained from 62 patients with hepatitis A, 100 patients with hepatitis B, 56 patients with hepatitis C, 52 patients with autoimmune hepatitis, 40 patients with rheumatoid arthritis, were tested for anti-HEV IgA, anti-HEV IgM and anti-HEV IgG (Table 1). Among the 410 patients who were not infected with HEV, including the 100 blood donors, anti-HEV IgM was positive in 7 serum samples, including 2 from patients with hepatitis, 4 from patients with autoimmune disorders. Anti-HEV IgA was detected in 6 serum samples including 4 from patients with hepatitis. The positivity for HEV antibodies could not be confirmed by the absorption test in any of the 10 samples. Serum samples obtained from 100 patients with sporadic acute hepatitis E were tested for the presence of IgM and IgA anti-HEV antibodies. All the 100 patients were positive for anti-HEV IgM and anti-HEV IgA. The presence of anti-HEV IgM and anti-HEV IgA was confirmed by the absorption test in the serum samples from all 100 patients. HEV IgA assay was highly specific and false- positive results in HEV-IgG and IgM assay could also be found in the IgA assay (Table 1).

Anti-HEV IgA, IgM and HEV-RNA were positive in patients with early HEV infection. HEV RNA remained detectable in the serum for one month, anti-HEV IgA for four months (> 50%, anti-HEV IgM for three months (> 50%), ALT and T-Bil for four months (Table 2).

DISCUSSION

Although the incidence of acute hepatitis especially acute hepatitis E has been gradually increased, the correct diagnosis of HEV infection largely depends on the sensitivity and specificity of HEV assay kits. The golden diagnosis standard for acute hepatitis E is based on detection of the HEV genome in serum or feces by RT-PCR, but it can only be used for earlier diagnosis. A solid-phase (sandwich or indirect) ELISA for detecting anti-HEV IgM and IgG is simple and has been currently used in diagnosis of HEV infection[24]. One of its disadvantages is the reduced sensitivity due to competition among virus-specific IgM, IgA and IgG for antigen-binding sites. Another potential disadvantage of the solid-phase test for IgM antibody is that IgM-rheumatoid factor in sera from patients with rheumatoid arthritis may produce a false-positive result. Although the existence of anti-HEV IgG in serum is very long, we cannot distinguish current acute infection from the last infection. It is important to find a new diagnosis assay. It has been reported that anti-HEV IgA can be utilized as an additional confirmatory antibody for recent HEV infection[25]. Although the presence of a specific antibody of IgA can show recent infection in several viral or nonviral diseases, including hepatitis A and B as well as cholera[26], the clinical and epidemiological significance of positivity for anti-HEV IgA remains to be fully verified.

In the present study, we used ELISA to detect IgA using HRP-labeled coat monoclonal anti-human IgA in stead of HRP-labeled coat monoclonal anti-human IgG. The results showed both anti-HEV IgM and anti-HEV IgA were detectable in serum samples obtained from all 60 patients tested who were subsequently diagnosed with HEV infection. The sensitivity was 100%. Among the 410 serum samples from subjects not recently infected with HEV, 6 were falsely positive for anti-HEV IgM and 5 were falsely positive for anti-HEV IgA, indicating that the false-positive rate is low both in anti-HEV IgM and in anti-HEV IgA. Except for one occult HEV infection, none was positive for both anti-HEV IgM and anti-HEV IgA. The specificity of anti-HEV IgA was verified in the absorbant and inhibitory assay.

HEV RNA, anti-HEV IgA, anti-HEV IgM, ALT and TBiL were defected in serum samples from 60 infected patients during follow-up. From these 60 patients, the serum samples obtained on d 15, 30, 60, 90 and 120 after the disease onset. The period of apparent manifestations was 40 d, liver disfunction usually lasted 5 mo, the peak of alanine aminotransferase and total bilirubin was 246 ± 53 U/L and 506 ± 123 μmol/L respectly. The positive number of HEV RNAs in serum was 60 (100%), 36 (60%), 3 (5%) and 0 in wk 2, mo 1-3 respectly. The positive number of anti-HEV IgA was 60 (100%), 60 (100%), 58 (97%), 56 (93%), 38 (63%), 18 (30%) in the second week, the first

1-5 mo after the disease onset, respectively. The positive number of anti-HEV IgM was 60 (100%), 60 (100%), 46 (77%), 34 (57%), 12 (20%), 2 (3%) in the second week, the first 1-3 mo, respectively. The positive rate of anti-HEV IgM and anti-HEV IgA was not significantly different in the first month after the disease onset. From the second month, the positive rate of anti-HEV IgM decreased sharply. The positive rate of anti-HEV IgA maintained at a high level. There were significant changes in them ($P < 0.05$). Since the duration of sero-positivity for HEV RNA, anti-HEV IgM and anti-HEV IgA lasted 1, 3 and 4 mo respectively, hepatic dysfunction lasted 4 mo, the diagnosis of HEV infection could be made by assay of anti-HEV IgM and anti-HEV IgA. Anti-HEV IgA and anti-HEV IgG can be used as a diagnostic indicator of HEV without anti-HEV IgM.

We have previously reported that indirect ELISA can detect anti-HEV IgG and anti-HEV IgA. The performance of IgA test has been extensively evaluated[27,28]. The assay specificity, sensitivity, and consistency are carefully documented. Based on the results obtained in the study, we conclude that anti-HEV IgA assay is as specific as the anti-HEV IgM assay in diagnosis of hepatitis E. IgM and IgA anti-HEV tests can be used together to characterize serum specimens with non-acute hepatitis E. Detecting both IgM anti-HEV and IgA anti-HEV at the same time help to minimize the false positive rate, thus improving diagnosis of HEV infection.

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RAPID COMMUNICATION

Effects of extract from Ginkgo biloba on carbon tetrachloride-induced liver injury in rats

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Abstract

AIM: To study the effects of extract from Ginkgo biloba (EGb) containing 22% flavonoid and 5% terpenoid on chronic liver injury and liver fibrosis of rats induced by carbon tetrachloride (CCl_4).

METHODS: All rats were randomly divided into control group, CCl_4-treated group, colchicine-treated group and EGb-protected group. Chronic liver injury was induced in experimental groups by subcutaneous injection of CCl_4 and fed with chows premixed with 79.5% corn powder, 20% lard and 0.5% cholesterol (v/v). EGb-protected group was treated with EGb (0.5 g/kg body weight per day) for 7 wk. At the end of wk 8, all the rats were killed. Liver function, liver fibrosis, oxidative stress and expression of transforming growth factor β_1 (TGF-β_1), a-smooth muscle actin (α-SMA) and type I collagens in liver were determined. In addition, pathology changes of liver tissue were observed under light microscope.

RESULTS: The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin (Alb) in EGb-protected group were notably improved as compared with the CCL_4-treated group ($P < 0.01$). The contents of serum hyaluronic acid (HA), type III procollagen (PCIII), type IV collagen (CIV) and the expression of hepatic tissue TGF-β_1, α-SMA and type I collagen in EGb-protected group were significantly lower than those in CCL_4-treated groups ($P < 0.05$, $P < 0.01$). The degrees of liver fibrosis in EGb-protected groups were lower than those in CCL_4-treated groups (6.58 ± 1.25 *vs* 9.52 ± 2.06, $P < 0.05$). Compared to the CCL_4-treated group, the levels of plasma glutathoine peroxidase (Se-GSH-Px), superoxide dismutase (SOD) and malondialdehyde (MDA) were strikingly improved also in EGb-protected group ($P < 0.05$, $P < 0.01$).

CONCLUSION: EGb resists oxidative stress and thereby reduces chronic liver injury and liver fibrosis in rats with liver injury induced by CCl_4.

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Key words: Rats; Hepatic fibrosis; Chronic liver damage; Extract from Ginkgo biloba; Lipid peroxidation

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INTRODUCTION

Chronic liver diseases commonly result in liver fibrosis and eventually liver cirrhosis. It has been demonstrated that oxygen-derived free radicals (ODFR) and lipid peroxidation play a critical role in the pathogenesis of various liver diseases including hepatic fibrosis[1-3]. Thereby it has become the key to prevent and cure hepatic damage by eliminating free radicals and preventing lipid peroxidation[4-8], and are applied in clinical medicine[9-11].

Extract from Ginkgo biloba (EGb) is an extract from dried ginkgo leaves. EGb could be used in the treatment of asthma and bronchitis. Recent studies have shown that flavonoid (ginkgo-flavone glycosides) and terpenoid (ginkgolides and bilobalides) are the most important active substances in the extract[12]. EGb is well known for its antioxidant property due to its ability to scavenge free radicals and to neutralize ferryl ion-induced peroxidation[13]. Furthermore, EGb can antagonize platelet-activating factor (PAF)[14]. Studies have shown that the antioxidant activity of EGb contributes to the prevention and treatment of diseases associated with oxidative stress[15-19]. These properties of the EGb are thought to provide many beneficial effects against chronic liver damage and liver fibrosis. Therefore, the purpose of this study was to investigate the protective effect of EGb on chronic liver damage and liver fibrosis induced by carbon tetrachloride (CCl_4) in rats.

MATERIALS AND METHODS

Animals and reagents

Male Spratgue-Dawley rats weighing 180 to 220 g were purchased from the Experimental Animal Center, Xi'an Jiaotong University Medical School (certification number: Shaanxi 8-005). Fifty-five animals were treated humanely according to the national guideline for the care of animals and divided randomly into four groups. The rats in control group were given common chows, while the rats in the other three groups were fed with chows premixed with 79.5% corn powder, 20% lard and 0.5% cholesterol (v/v). Meanwhile, 3 mL/kg body weight CCl_4 (analytically pure, Xi'an Chemical Factory, China) as a solution 40% (v/v) in peanut oil was injected subcutaneously twice a week into the rats of CCl_4-treated group, and the first dosage was 5 mL/kg body weight CCl_4. The EGb-treated group and colchicine-treated group received EGb (Tianbao Yinxingpian, 19.2 mg flavonoid glycoside in 80 mg EGb of each tablet, Guizhou Xinbang Drug Design Co. Ltd, China) 0.5 mg/kg per day and colchicine (Xi'an Chemical Factory, China), 0.5 g/kg body weight per day. The control group and CCl_4-treated group were fed with distilled water.

Rats were sacrificed 7 wk later. Serum or plasma was collected and stored at -20℃ for determination. Specimens of isolated rat livers were fixed in formaldehyde and stored in liquid nitrogen.

Detection of serum markers

Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and concentration of album (Alb) were assayed by a 7170-A automatic analyzer (Hitachi, Japan). Meanwhile, serum hyaluronic acid (HA), type III procollgen (PCIII) and type IV collagen (CIV) concentrations were measured radioimmunologically using commercial kits (Peking Northern Biotech Institute, China).

Parameters of plasma antioxidation

The activity of glutathoine peroxidase (GSH-Px) and superoxide dismutase (SOD) in plasma was quantified, and the concentration of malondialdehyde (MDA) in plasma was determined as previously described[20].

Liver histopathology

For histopathological analysis, thin liver slices were cut, fixed in formaldehyde and embedded in paraffin. Five-μm thick sections were then stained with hematoxylin-eosin (HE) and masons trichrome before observed under light microscope. The degree of hepatic fibrosis was determined by a semi-quantitative method[21].

Immunohistochemical examination

Liver tissue sections were mounted onto slides, deparaffinized in xylene, and rehydrated in alcohol. The levels of transforming growth factor β_1 (TGF-β_1) (Dako, USA), α-smooth muscle actin (α-SMA) (Neomarkers, USA) and type I collagen (Sigma, USA) were determined by immunohistochemical methods using S-P immunohistochemical test kits (Maixing Bio-technological Co., Ltd, Fujian, China). Based on the histological staging, α-SMA, type I collagen and TGF-β_1 positive cells were expressed as percentage of the total area of the specimen.

Table 1 Effect of EGb on liver function in rats with CCl_4-induced injuries (mean ± SD)

Group	*n*	ALT (nkat•L^{-1})	AST (nkat•L^{-1})	Alb (g•L^{-1})
Control	10	42.8 ± 12.5	112.5 ± 36.5	35.3 ± 3.8
CCl_4 treated	11	286.9 ± 52.6^b	250.4 ± 22.5^b	25.5 ± 4.8^b
Colchicines	12	165.2 ± 36.5^d	193.6 ± 20.8	29.5 ± 4.1
EGb	12	92.5 ± 27.5^d	118.2 ± 18.2^d	32.3 ± 6.2^d

$^bP < 0.01$ *vs* control group; $^dP < 0.01$ *vs* CCl_4-treated group.

Table 2 Effect of EGb on liver antioxidation in rats with CCl_4-induced injuries (mean ± SD)

Group	*n*	SOD (nkat•mg^{-1})	GSH-Px (nkat•g protein^{-1})	MDA (nmol•g^{-1})
Control	10	336.9 ± 82.4	482.4 ± 119.3	62.5 ± 15.6
CCl_4 treated	11	51.5 ± 12.8^b	162.5 ± 46.8^b	323.5 ± 122.1^b
Colchicines	12	112.5 ± 67.5^d	178.2 ± 78.5^d	168.3 ± 66.4^d
EGb	12	165.2 ± 76.1^d	303.6 ± 101.8^a	112.3 ± 33.9^a

$^bP < 0.01$ *vs* control group; $^dP < 0.01$, $^aP < 0.05$ *vs* CCl_4-treated group.

Statistical analysis

Data were expressed as mean ± SD unless indicated specially, and analyzed by software package SPSS. The Mann-Whitney *u* test for nonparametric and unpaired values, Student's *t*-test or Fisher's exact test was used as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Changes of serum indicators

Table 1 gives the values for the activities of serum indicator enzymes and Alb. CCl_4 produced a marked increase in the activities of serum ALT and AST and a significant decrease in Alb level. CCl_4 plus EGb-protected group showed a significant decrease in enzyme levels and a significant increase in Alb level.

Effects of EGb on plasma antioxidation in rats

The activities of plasma SOD and GSH-Px were markedly reduced in CCl_4-treated group compared with control group, while the content of MDA was significantly increased ($P < 0.01$). Markers in EGb-protected group were dramatically recovered compared with model group ($P < 0.01$, Table 2).

Effects of EGb on serum fibrosis

The contents of HA, PCIII and CIV were significantly increased in CCl_4-treated group compared with control group ($P < 0.01$). EGb could reduce the three ECM markers ($P < 0.05$, $P < 0.01$, Table 3).

Influence of EGb on degree of liver fibrosis in rats

HE and mason's trichrome-stained liver sections were observed. The normal control livers showed normal lobular architecture with central veins and radiating hepatic cords

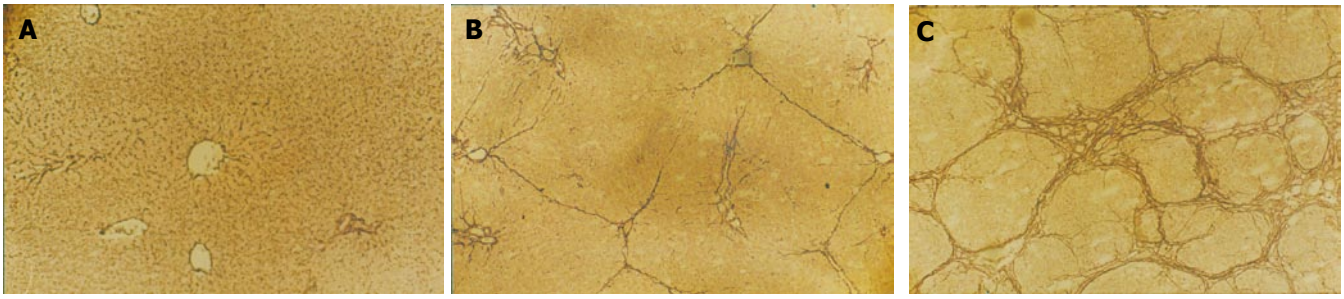


Figure 1 Effects of EGb on histology of CCL_4-induced fibrotic rat liver. Masson trichome staining, scale bar = 40 μm, original magnification × 100. **A**: Normal rat liver; **B**: Fibrosis in CCL_4-treated group; **C**: Fibrosis in EGb-protected group.

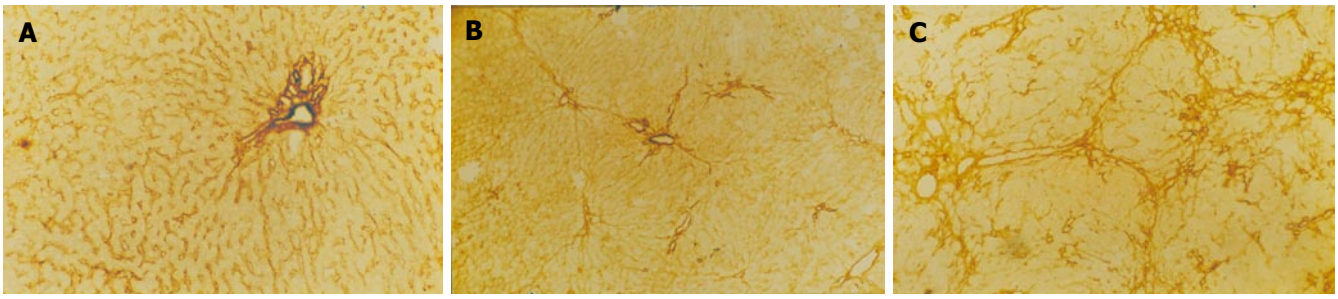


Figure 2 Effects of EGb on type I collagen expression in CCl_4-induced fibrotic rat liver. Immunohistochemistry staining, scale bar = 40 μm, original magnification × 100. **A**: Normal rat liver; **B**: Strong expression of type I collagen in CCL_4-treated group; **C**: Expression of type I collagen in EGb-protected group.

Table 3 Effect of EGb on serum levels of HA, PCIII and CIV (mean ± SD)

Group	*n*	HA (μg•L^{-1})	PCIII (μg•L^{-1})	CIV (μg•L^{-1})
Control	10	40.5 ± 7.8	15.3 ± 3.2	18.5 ± 4.0
CCl_4 treated	11	126.9 ± 12.8[a]	36.5 ± 7.2[a]	56.2 ± 7.8[b]
Colchicines	12	79.2 ± 16.5[b]	23.5 ± 5.6[b]	33.6 ± 5.5[d]
EGb	12	91.5 ± 11.6[b]	28.5 ± 6.0[a]	38.4 ± 5.8[a]

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$, [a]$P < 0.05$ *vs* CCl_4-treated group.

Table 4 Effect of EGb on degree of liver fibrosis in rats (mean ± SD)

Group	*n*	Degree of liver fibrosis			
		0	I	II	III
Control	10	10	0	0	0
CCL_4 treated[b]	11	0	1	2	8
EGb[a]	12	0	6	4	2

[b]$P < 0.01$ *vs* control group; [a]$P < 0.05$ *vs* CCl_4-treated group.

(Figure 1A). Histological examination of livers from CCL_4-treated group rats revealed hepatocyte denaturation, necrosis, connective tissue hyperplasia, hepatic lobule disorganization and pseudo-lobule formation, degree III fibrosis and hyperplasia (Figure 1B). However, in EGb-protected group, the hepatocyte denaturation and necrosis were not obvious. Hyperplasia of connective tissue was decreased. Liver fibrosis belonged to degree I or II (Figure 1C), but pseudo-lobule formation was not found.

The fibrosis degrees of all groups are shown in Table 4. In addition, detection of hepatic fibrosis markers in serum (6.58 ± 1.25) was also lower than that in CCL_4-treated group (9.52 ± 2.06) compared with control group ($P < 0.05$).

Effects of EGb on TGF-β$_1$, α-SMA, type I collagen expression in rats with CCl_4-induced injuries

Immunohistochemistry revealed that type I collagen was stained in vascular smooth muscle cells and sinusoids of control group rat livers only (Figure 2A). While in CCl_4-treated group, type I collagen was strongly expressed within centrilobular and periportal fibrotic bands (Figure 2B). The percentage of type I collagen expressing area in EGb-protected group was obviously lower than that in CCl_4-treated group ($P < 0.05$, Figure 2C).

The percentage areas of type I collagen, TGF-β$_1$ and α-SMA staining in the rat liver are shown in Figure 3. The results suggested that administration of CCl_4 significantly increased the percentage areas of all the three-marker staining. However, this condition could be significantly suppressed by use of EGb.

DISCUSSION

Carbon tetrachloride (CCl_4) is widely used to experimentally induce liver damage. Peroxidation of membrane lipids secondary to the formation of trichloromethyl ($CCl_{3\ 1/2}$ and /or $CCl_3OO_{1/2}$) radicals is believed to be the basis for the toxic effect of CCl_4[22]. These radicals are capable of initiating a chain of lipid peroxidation reactions by abstracting hydrogen from polyunsaturated fatty acids (PUFA). Peroxidation of lipids, particularly those containing PUFA, can dramatically change the properties of biological membranes, resulting in severe cell damage and play a significant role in pathogenesis of diseases. Membrane lipid peroxidation induces crosslinking in proteins. This can be provoked by the MDA generated

during PUFA degradation. The two-aldehyde functions of MDA can covalently bind to the free amine functions of proteins and lead to an irreversible polymerization. Oxidative damage can also produce reversible disulfide-bonded polymers resulting from the oxidation of protein tiols. These substances lead to CCl_4 hepatoxicity by starting lipid peroxidation in the membranes[23-24].

Standard Ginkgo biloba extract, EGb 761 (commercial name), contains 22%-27% flavonoids (ginkgo-flavone glycosides) and 5%-7% terpenoids (ginkgolides and bilobalides), which are the most important active substances in the extract. The most important flavonoids are glycosides of kaempferol, quercetin, and isorhamnetin with glucose or rhamnose. Ginkgolides, not found in any other living species and only presented in Ginkgo biloba extract, can be divided into types A, B, C, and a very small quantity of J, which are only different in the number and position of hydroxyl groups[12,13,25]. Several mechanisms of action of EGb have been described: vasoregulatory activity and rheological effects, such as decreased iscosity, anti-platelet activating factor activity, and metabolic changes, such as increase neuron tolerance to anoxia, as well as gene- regulatory effects, such as suggesting anticancer activity, and prevention of damage to cell membranes caused by free radicals[13-16, 26].

Recent studies have provided considerable support for the *in vitro* and *in vivo* protective effects of EGb 761 on ischemia/reperfusion injury or oxidative stress[13,15,19,27]. These effects are closely related to the ability of ginkgo-flavone glycosides, the main component of EGb. It has been reported that flavonoids origin nuclear can reduce hydroxy function group, which could capture oxygen-derived free radicals, such as superoxide anion, hydroxyl and peroxyl radicals, and nitric oxide, act as a donator of hydrogen atom to terminate pathological aggravation of free radical chain reaction and lipid peroxidation, and relieve the injury caused by oxygen-derived free radicals and lipid peroxidation[12,13]. Consequently, it reduces lipid peroxidation. Thus EGb has wide antioxidant effects and can be used in disease treatment[16-19,25,28].

Recent studies indicate that oxidative stress is relevant to the formation of fibrosis in most chronic liver diseases, frequently accompanying decline of anti-oxidation abilities, such as accumulation of lipid peroxidation, descent of anti-oxidase capability, decline of reduced glutathione, which plays an important role in pathologenesis of various liver diseases. Therefore, lipid peroxidation is an important factor, which connects inflammatory injury with hepatic fibrosis. Under the circumstances, we suppose that antioxidant therapy could protect hepatocytes from lipid peroxidation, and prevent or relieve chronic liver damage processes[1-3,22]. Daba *et al*[22] demonstrated that EGb is a protective agent against lung toxicity induced by BLM treatment. This study showed that the levels of hepatic enzyme indicator MDA were decreased while the serum levels of ALB and the activity of SOD and GSH-Px in the CCl_4 plus EGb-protected group were decreased when compared with CCl_4-treated group. The findings are similar to the results of Daba *et al*[22], which further confirmed that EGb can inhibit CCl_4-induced lipid peroxidation in plasma and liver tissue and has protective effect on experimental chronic liver damage induced by CCl_4.

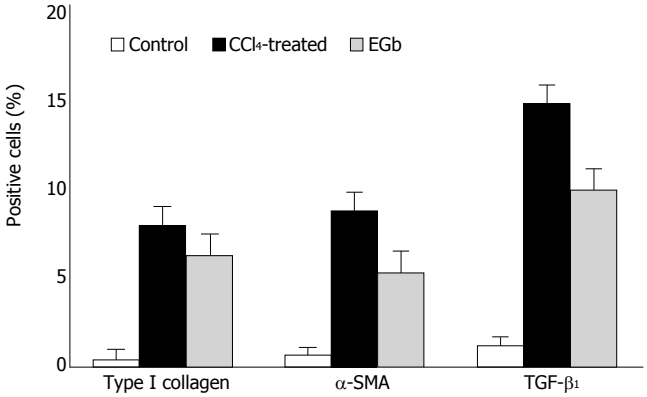


Figure 3 Percentage areas (%) of type I collagen, α-SMA, and TGF-β1 in rat liver.

Hepatic fibrosis is usually initiated by hepatocyte damage, leading to recruitment of inflammatory cells and platelets, activation of kupffer cells and subsequent release of cytokines and growth factors[29,30]. These factors probably link the inflammatory and repair phase of liver cirrhosis by activating hepatic stellate cells (HSC)[31,32]. Recently, HSC have been identified as an important cellular source of extracellular matrix (ECM) in liver fibrosis[33]. These cells reside in the perisinusoidal space of Disse that separates hepatocytes from the sinusoidal endothelium. Upon injury (e.g. by toxins or chronic hepatitis), the normally quiescent HSC are activated and start to proliferate. The activated HSC undergo a phenotypic transdifferentiation to contract myofibroblasts (MFB) expressing α-SMA, and produce an excess of ECM molecules. TGF-β1 is one of the most fibrogenetic cytokines on HSC, and initiates HSC activation characterized by proliferation and expression of α-SMA and ECM[34-36]. It has been shown that lipid peroxidation, free-radical-mediated process, and certain lipid peroxidation products induce genetic overexpression of fibrogenic cytokines (e.g. TGF-β1 and platelet-derived growth factor) and increase the synthesis of collagen. Free radicals and MDA can stimulate the synthesis of collagen and initiate the activation of HSC[37,38]. Our results showed that the expression of TGF-β1, α-SMA and type I collagen in liver, the level of hepatic fibrosis markers in serum and the degrees of fibrosis in liver tissue in EGb-protected group were obviously lower than those in model group, accompanying the decreasing MDA content. These findings suggest that EGb can suppress TGF-β1 expression and TGF-β1-initiated HSC activation, inhibit HSC proliferation and down-regulate α-SMA protein. The antifibrotic effect of EGb may be in part due to its radical scavenging action or antioxidant activity.

In conclusion, EGb is effective against oxidative liver damage and liver fibrosis induced by CCl_4, the protective effect may be due to its radical scavenging action or antioxidant activity. Therefore, EGb can be used to prevent and treat hepatic fibrosis. Further investigations on this matter are needed.

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RAPID COMMUNICATION

Functional studies of a novel oncogene TGM3 in human esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the role of transglutaminase 3 (TGM3) gene in human esophageal squamous cell carcinoma (ESCC), and analyze its relationship with clinicopathological parameters.

METHODS: Gene expression of TGM3 in fresh esophageal cancer tissues and their corresponding normal mucosas was detected immunohistochemically(IHC) by means of tissue microarray(TMA). Its correlation with clinical characteristics was evaluated and analyzed by univariate analysis. All statistical analyses were performed by SPSS version 10.0.

RESULTS: Expression rate of TGM3 was reduced to 81.8% in ESCC. Expression of TGM3 was significantly inversely correlated with histological grade of esophageal carcinoma (in grade I, II and III tumors, the reduced expression was 4/7, 57/71, and 20/21, respectively, $P < 0.05$), whereas it had no obvious correlations with lymph node metastasis and depth of invasion.

CONCLUSION: Reduced expression of TGM3 may play an important role in esophageal carcinogenesis.

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Key words: Esophageal squamous cell carcinoma; Immunohistochemically; Tissue microarray

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INTRODUCTION

Esophageal cancer ranks among the 10 most frequent cancers in the world, with a predominant distribution in developing countries. Our previous study showed that genetic susceptibility to esophageal cancer was one of the important causes for the high prevalence and familial aggregation of this disease in some areas of northern China[1]. Chen *et al* observed the down-regulation of TGM3 gene in human ESCC using polymerase chain reaction (RT-PCR) and Northern blot techniques[2]. To our best knowledge, this is the first time to investigate the protein expression of TGM3 in ESCC. The expression levels of TGM3 gene decreased in ESCC tissues compared with their normal counterparts. The results are consistent with that from RT-PCR and Northern blot. Therefore, TGM3 gene might be related to human ESCC and further study may provide insight into the mechanisms of carcinogenesis of esophagus. TGM3 is a member of a family of Ca^{2+}-dependent enzymes, which can catalyze the substrates to form a complex that is related to the differentiation of the stratified squamous epithelia[3]. The gene is localized on chromosome 20q11.2[4]. The full-length cDNA of TGM3 is about 2.6 kb, encoding 692 amino acids. TGM3 as a proenzyme, whose molecular weight is about 77 ku, becomes highly active upon proteolytic cleavage into 50 ku (amino-terminal) and 27 ku species[2].

Tissue microarray was first introduced in 1998[5], which allows simultaneous analysis of many tumors using small-diameter cores sampled from larger blocks of tissue, but may be limited by tumor heterogeneity[6]. This study used TMA for the study of four molecules of interest as prognostic factors in ESCC, including methods for assessing immunocytochemical scoring of microarrays. TMA blocks were constructed from 99 cases of ESCC with corresponding normal tissues, taking four cores from different areas of each tumor and adjacent esophageal epithelia. Immunocytochemical labelling was performed for TGM3. The extent and intensity of scoring was determined for each core and the degree of agreement was determined for results from the assessment of two, three or four cores for each carcinoma. In a subset of 99 representative cases, the labelling in the TMA was compared with that in whole-tissue sections of the

same carcinomas. An adequate sample of carcinoma was obtained in more than 90% of the 396 cores. TMA is a reliable tool to demonstrate cellular and molecular alterations in ESCC. We recommend using the mean results from four cores for biological studies, with analysis of categorical data based on quartile groups.

MATERIALS AND METHODS

Materials

Specimens of cancer tissues and matched adjacent normal mucosa were taken from 99 consecutive patients with squamous cell carcinoma of the thoracic esophagus who had undergone esophagectomy with regional lymph node dissected from Oct 2004 to Sept 2005 at the Department of Thoracic Surgery, the First Affiliated Hospital of Anhui Medical University. None of them received irradiation or chemotherapy preoperatively. The patients included 87 men and 12 women with a mean age of 54 (range 36-79) years. Six tumors were located in the upper thorax, 61 in the middle thorax and 32 in the lower thorax (Table 1). The removed specimens were examined histologically with hematoxylin and eosin staining, and then the clinicopathologic stage was determined according to TNM classification.

Table 1 Clinical and histopathological characteristics of patients

Characteristics	*n* = 99 (%)
Sex	
Male	87 (87.9)
Female	12 (12.1)
Location of tumor	
Upper thoracic	6 (6.1)
Middle thoracic	61 (61.6)
Lower thoracic	32 (32.3)
Histological grade	
Ⅰ	7 (7.1)
Ⅱ	71 (71.7)
Ⅲ	21 (21.2)
Depth of invasion	
T1	5 (5.1)
T2	35 (35.4)
T3	59 (59.6)
T4	0 (0)
Lymph node metastasis	
Positive	32 (32.3)
Negative	67 (67.7)

Construction of TMA

Archival paraffin-embedded, formalin-fixed tissues are collected. Two pathologists selected representative areas from each donor tumor block, and then punched cores 0.6 mm in diameter, from the donor block. Sections of the resultant tissue microarray were sliced and transferred to glass slides for processing of TGM3 by immunohistochemistry (Pictured is a 120-specimen TMA with 0.6 mm spots).

Immunohistochemical staining

Immunohistochemical analysis was done retrospectively. Resected esophageal specimens, including both tumor and normal mucosae, were fixed in a 40 g/L formaldehyde solution and embedded in paraffin. The following antibody was used in this study: goat polyclonal anti-human TGM3 antibody (Santa Cruz Biotech Co, USA) diluted 1:100 in PBS. Four μm thick sections of formalin-fixed paraffin-embedded tissue blocks of esophageal tumors were sliced. TMAs were deparaffinized, dehydrated and blocked to remove endogenous peroxidase activity by 3 mL/L H_2O_2 in methanol for 30 min. TMAs were treated with microwave in 0.1 mol/L citrate buffer pH 6.0 at 96℃ for 15 min. After incubation with 100 mL/L normal goat serum to block non-specific binding, they were then incubated with the primary antibodies overnight at 4℃. After antibody was washed with PBS, TMAs were incubated with the secondary antibody and the third antibody (Streptavidin/HRP) according to the manufacturer's instructions, and finally DAB was visualized. Tissues were counterstained with hematoxylin. Negative control was designed by using PBS instead of primary antibody.

Evaluation of staining

The cytoplasmic expression of TGM3 in the ESCC was evaluated as described previously and scored as follows: (a) 0, < 5% of the epithelial cells in the respective lesions; (b) 1, 5%-25% of the epithelial cells in the respective lesions; (c) 2, 26%-50% of the epithelial cells in the respective lesions; (d) 3, 51%-75% of the epithelial cells in the respective lesions; and (e) 4, >75% of the epithelial cells in the respective lesions. The intensity was graded as follows: (a) 0, negative; (b) 1+, weak; (c) 2+, moderate; and (d) 3+, strong (as intense as normal mucosa). A final score between 0 and 12 was achieved by multiplication of the extent of positivity and intensity[7,8]. Scores of 8-12 were defined as "strong expression", scores of 3-7 were defined as "reduced expression", and scores of 0-3 were defined as "markedly reduced expression". Positive staining more than 5% in cell cytoplasm was defined as positive staining; less than 50% in cell cytoplasm was defined as reduced expression; more than 50% in cell cytoplasm was defined as preserved expression[9].

Statistical analysis

χ^2 test or Fisher's exact probability test and Spearman rank correlation coefficient analysis were used to assess the association between immunohistochemical features and clinicopathological characteristics. A P value less than 0.05 was considered statistically significant. All the statistical analyses were performed using the SPSS 10.0 V for Windows.

RESULTS

Expression of TGM3 in esophageal squamous cell carcinoma

TGM3 positive expression showed brown stained signals in the normal mucosa cytoplasm and reduced or positive expression in ESCC, with only a small number of expressions in cell membranes. No nuclear expression of TGM3 was observed in cells. The positive expression rate of TGM3 in 99 esophageal cancer patients was 60.6% (60/99). Significant positive correlation was found in TGM3 expression of the cases between paired normal

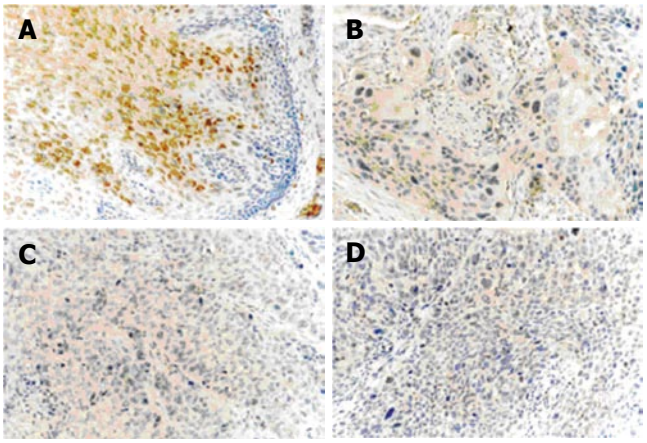


Figure 1 Immunohistochemical analysis of TGM3 in paired ESCC tissue samples using anti- TGM3 antibody (1:100). Diffuse and strong staining was detected in the cytoplasm of the normal epithelial cells (**A**), while sporadic and weak staining was observed in the cytoplasm of matched esophageal cancer epithelial cells (**B**: well-differentiated, **C**: moderately-differentiated, **D**: poorly-differentiated) (original magnification × 200).

and cancerous tissue of esophageal carcinoma ($P < 0.05$). The reduced expression rate of TGM3 was 81.8% (81/99) (Figure 1).

Relationship between TGM3 expression and clinicopathologic variables in esophageal squamous cell carcinoma

Expression of TGM3 correlated significantly only with histological grade of esophageal squamous cell carcinoma. Significant inverse correlation existed between the intensity of TGM3 expression and histological grade ($P < 0.05$). No significant correlation was found between abnormal expression of TGM3 and lymph node metastasis and depth of invasion (Table 2).

Table 2 Relationship between clinicopathological parameters and expression of TGM3

Type	Cases	TGM3		*P*
		Preserved	Reduced	
Histological grade				
Ⅰ	7	3	4	< 0.05
Ⅱ	71	14	57	
Ⅲ	21	1	20	
Depth of invasion				
Mucous layer	5	0	5	> 0.05
Muscular layer	35	5	30	
Full-thickness	59	13	46	
Lymph node metastases				
Positive	32	6	26	> 0.05
Negative	67	12	55	

DISCUSSION

Transglutaminase(TGM) enzymes are widespread in both plants and animals[10,11]. They catalyze the formation of anisodipeptide cross-linking between the ε-NH2 side chain of a protein-bound lysine residue and the γ-amide side chain of aprotein-bound glutamine residue, thereby forming an insoluble macromolecular aggregate that is used for a variety of cellular functions. To date, there are nine known TGM enzymes encoded in the human genome[12], and interestingly, three of them are active in the epidermis and its appendages. These include: the TGM1 enzyme[13] which can function as membrane-associated[14], soluble full-length, and soluble proteolytically activated processed forms in the epidermis[15]; the soluble, "tissue" TGM2 enzyme[16], and the soluble TGM3 proenzyme, which also requires proteolytic activation[17,18]. The TGM3 enzyme is expressed during the late stages of the terminal differentiation of the epidermis and in certain cell types of the hair follicle[19]. The enzyme is thought to be critically involved in the cross-linking of structural proteins and in the formation of the cornified cell envelope, thereby contributing to rigid structures that play vital roles in shape determination and/or barrier functions[20-22]. Although TGM3 mRNA represents less than 2% of the TGM transcripts, the activated TGM3 accounts for up to 75% of the total TGM activity in mammalian epidermis[15].

The understanding of the molecular basis of tumor development has progressed dramatically in the last two decades. It is well known that tumor is essentially a genetic disease. Therefore it is important to demonstrate what these oncogenes are and how they work in carcinogenesis. Identifying the genetic differences between normal and tumor cells or tissues will help discover the genes that directly cause tumor or are associated with tumorigenesis and provide novel markers for early detection and appropriate therapy.

Although the role of TGM3 has been well established in skin keratinocytes, little information is available concerning its involvement in esophageal epithelia. In previous study, TGM3 gene showed down-regulation in human ESCC tissues[2,23]. To verify this differential expression, we first investigated immunohistochemically the expression of TGM3 protein in paired ESCC by means of TMA. The results of IHC revealed that TGM3 reduced expression in 81.8% (81/99) examined tumor tissues relative to the corresponding normal tissues. Among 99 esophageal tumors examined, which were histologically squamous-cell carcinomas, seven tumors were in grade I, 71 were in grade II and 21 were in grade III. Our data showed that decreased TGM3 expression was uncommon in 3 phases of primary esophageal cancer. This is in agreement with previous studies on a variety of cancers, such as laryngeal carcinoma[24], head and neck squamous cell carcinoma[25], oral squamous cell carcinoma from leukoplakia[26]. In all these studies, reduction or loss of TGM3 expression was significantly associated with dedifferentiation, increased invasiveness, and high incidence of lymph node metastasis, hematogenous recurrence and poor prognosis in a number of human carcinomas. In our investigation, 81.8% of ESCC showed reduced expression of TGM3. As a marker associated with squamous cell histological grade, the level of TGM3 expression had an inverse correlation with histological grade, but not with lymphatic metastases and depth of tumor invasion. TGM3, therefore, is one of the most important adhesion molecules expressed by epithelial cells and is regarded as an invasion suppressor molecule. Further investigations are needed to predict the prognostic value of TGM3 for esophageal tumor patients.

ACKNOWLEDGMENTS

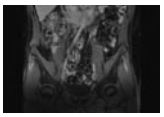
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CASE REPORT

Mesenteric adenitis caused by *Yersinia pseudotubercolosis* in a patient subsequently diagnosed with Crohn's disease of the terminal ileum

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Abstract

Although the association between inflammatory bowel disease and gastrointestinal infections has been suggested, the mechanisms involved in the pathogenesis of Crohn's disease (CD) are still undetermined. We report the case of a man, who presented with mesenteric adenitis initially due to a *Yersinia pseudotubercolosis* infection, who was later diagnosed with Crohn's disease. This case is in keeping with recent evidence in the literature which suggests that CD is a disease linked to abnormal immune responses to enteric bacteria in genetically susceptible individuals.

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Key words: *Yersinia pseudotubercolosis*; Crohn's disease; Ileitis

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INTRODUCTION

Infection by *Yersinia pseudotubercolosis* is of increasing pathological importance. Yersinial subspecies should be considered as etiological agents in gatrointestinal infections. Patients with mesenteric adenitis normally present with symptoms similar to those of appendicitis. Such non complicated infections are, at present, successfully managed with systemic antimicrobial agents[1].

We report the case of a young man with terminal ileitis due to *Yersinia pseudotubercolosis* infection, in whom Crohn's disease (CD) was subsequently diagnosed.

CASE REPORT

A 29 year old man was admitted to our GI unit with abdominal pain and diffuse arthralgies. He had demonstrated 5 kg weight loss in the last 7 months, which was probably due to a reduction in food intake because of abdominal symptoms. There was no family history of inflammatory bowel disease or cancer and no previous abdominal surgery. In the preceeding few months the patient had suffered from episodes of severe abdominal pain, emesis, fever (38-40℃), articular and back pain and demonstrated elevated inflammatory markers. The clinical condition of the patient remained poor despite antimicrobial treatment (ceftriaxone) and one month later he developed pleuropericardic effusion and active synovitis of the right knee.

An ultrasound examination revealed thickening of the last 20 cm of the terminal ileum (5-6 mm) with light retrodilatation and pathologically enlarged lymph nodes (1-2 cm). An abdominal MRI confirmed the wall thickening and the wall post iv contrast material enhancement, expression of active inflammation, and the presence of round and excavated mesenteric lymph nodes (Figure 1). Due to persistent abdominal pain, a laparoscopy was performed and biopsies of two mesenteric lymphnodes (transvers mesocolon) were collected. These revealed no histologic evidence of lymphoma. The diagnosis of *Yersinia pseudotubercolosis* infection was confirmed by serology and bacterial culture of the biopsied material. On the basis of suspected ileitis due to Yersinia pseudotubercolosis, cotrimoxazole and cefazime therapy was started. However, despite the antimicrobial treatment, intestinal and arthralgies were still present. Physical examination of the patient revealed poor condition. The abdomen was tender, with pain at palpation of the right iliac region. The tympanites was enhanced. Peristalsis was reduced with metallic sound.

There were no signs of immunodeficiency and most laboratory tests were normal, but indexes of inflammation were present (increased white blood cell count :14, 8/mm^3;

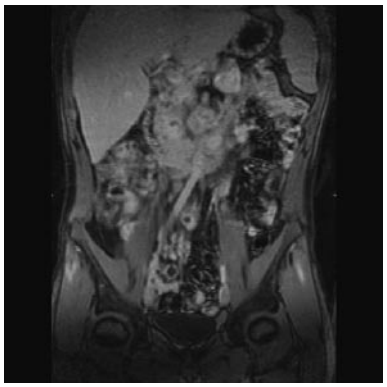


Figure 1 MRI coronal view, T1-weighted post oral administration of superparamagnetic contrast agent. Image illustrates marked wall thickening and enhancement at the level of terminal ileum and enlarged mesenteric lymph nodes.

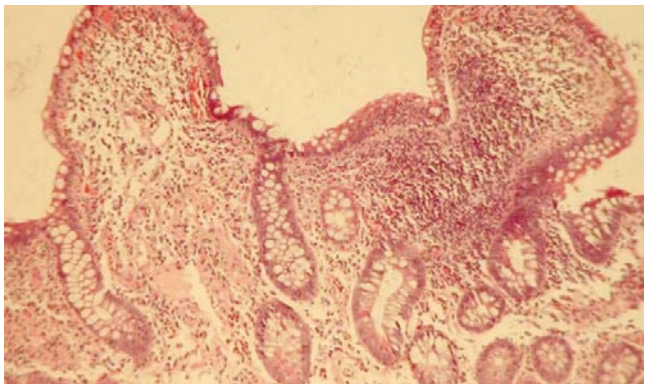


Figure 2 Biopsy of the terminal ileum showing histopathological findings of Crohn's disease.

neutrophils 74%, ESR 60 mm/1 h; CRP 8,14 mg/dL) with associated anemia (HB 118 g/L; ferraemia 21,3 mcg/dL).

The presence of mesenteric lymphoadenitis and anemia suggested Coeliac disease as a possible diagnosis. However, upper gastrointestinal endoscopy and serological screening (EMA IgA, EMA IgG, antibodies anti-transglutaminase IgA) were all negative. Finally, a total colonoscopy with retrograde ileoscopy was performed. It showed erythema, edema, multiple erosions and ulcerations in the ileum. Random biopsies of the ileum were taken and the histopathological findings showed epithelial damage, distorsions of villous architecture and increased inflammatory infiltrate (lymphocytes and eosinophils) in the lamina propria and submucosa (Figure 2). These findings suggested a diagnosis of Crohn's disease, treatment with oral mesalazine 2, 4 g/d was started and clinical improvement ensued. This improvement may also have related to a delayed effect of previous administration of systemic antibiotics. After one year of follow-up the patient was still in remission, and repeated endoscopy and ileal biopsies confirmed the diagnosis.

DISCUSSION

This case report describes the development of Crohn's disease in a patient with a previous *Yersinia pseudotubercolosis* infection. In recent years, very little has been published on the relationship between Yersinia infections and Crohn's disease[2,3], although microorganisms have long been suspected of causing CD. *Yersinia pseudotubercolosis* is a Gram-negative coccobacillus and a primary pathogen of wild and domestic animals and birds. It is an enteric pathogen that causes a variety of clinical symptoms in the human. These include diarrhoea, abdominal pain and post-bacteremia systemic symptoms such as fever, scarlattiniform skin rash, conjunctivitis, erythema nodosum and lymphadenophathy. Additional complications in the form of reactive arthritis, acute uveitis, coronary aneurysm and acute renal failure have also been reported[4].

When refrigerated, this organism survives while others do not. As a result, in a recent paper Hugot et al., have suggested the refrigerator as a new contributing factor in the development of CD[5]. In relation to the observation that CD is linked to a familial environmental risk factor related to modern lifestyle, the authors show temporal and geographical coincidences between the development of the refrigerator and the outbreak of CD.

This bacterium exhibits a marked tropism for lymphatic tissue and the characteristic histopathological findings in mesenteric lymph nodes suggest that the stimulation of T cells play a central role in the pathogenesis of this infection[6]. A key factor in the etiology of IBD is the chronic inflammatory process. Much evidence suggest that the transcription factor nuclear factor-kappa B (NFkB) plays an essential role in developing inflammation through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as cyclooxigenase 2 (COX2) and inducible nitric oxide synthase (iNOS)[7]. Evidence has shown that Gram negative bacteria such as Yersinia pseudotubercolosis, target the eukaryotic signal transduction pathways that lead to NFkB activation[8]. NOD2 (renamed CARD15 by the International Nomenclature Committee) a gene involved in innate immunity, is an activator of the NFkB pathway[9]. The link between bacterial components, including lipopolysaccharide (LPS) and CD is strongly supported by the discovery that mutations in CARD15 predispose people to the disease[10]. Recent studies have demonstrated that a frameshift mutation in CARD15 may result in innate hyporesponsiveness to bacterial components, inducing an altered adaptive response to enteric bacteria[11].

This report supports previous evidence that CD is a disease of an abnormal immune response to enteric bacteria in genetically susceptible individuals. In common with previous studies associating infectious organism with CD, much work remains to elucidate whether the presence of the microorganisms is an epiphenomenon or actually a factor in the pathogenesis of CD.

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CASE REPORT

Actively bleeding Dieulafoy's lesion of the small bowel identified by capsule endoscopy and treated by push enteroscopy

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Abstract

Dieulafoy's lesion is an unusual cause of recurrent GI bleeding. This report describes a case of actively bleeding Dieulafoy's lesion of the small bowel in which the diagnosis was made by capsule endoscopy, followed by treatment with the use of push enteroscopy. The case illustrates that capsule endoscopy and enteroscopy are highly complementary in patients with small bowel diseases.

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Key words: Dieulafoy's lesion; GI hemorrhage; Small bowel; Capsule endoscopy; Push enteroscopy

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INTRODUCTION

Dieulafoy's lesion (DL) is an unusual cause of recurrent gastrointestinal (GI) bleeding[1-3]. It consists of a large-caliber, tortuous artery, 1-3 mm in diameter, that lies in the submucosa in close contact with the mucosa over a variable distance. Massive bleeding can occur with erosion of the mucosa and arterial wall[4] .

Because of the small size of the lesion and the normal surrounding mucosa, the diagnosis of a Dieulafoy's lesion can be made with confidence only during active bleeding. A variety of endoscopic modalities have been used, either alone or in combination, with high success rates. This report describes a case of actively bleeding Dieulafoy's lesion of the small bowel in which the diagnosis was made by capsule endoscopy (CE), followed by treatment with the use of push enteroscopy.

CASE REPORT

A 50-year-old man was admitted to our hospital with chief complain of melena. He had a 1-year history of prosthetic heart valves implantation and was taking coumadin. At admission, Hb level was 83 g/L, hematocrit 21.5%, red blood cell count 2.15×10^{12}/L, a platelet count 249×10^{9}/L and international normalized ratio (INR) 3.35. However, the patient was hemodynamically stable. Esophagogastroduodenoscopy (EGD) and colonoscopy, performed on emergent basis, were negative for a source of bleeding. Hence, capsule endoscopy was performed because it was necessary to rule out other possible sources of bleeding, which revealed a single active bleeding vascular lesion of the small bowel (Figure 1). Peroral push enteroscopy disclosed a single oozing vessel with normal surrounding mucosa and no evidence of ulceration (Figure 2).

A hemostatic clip (Quick-Clip; Olympus Optical Co. Ltd., Tokyo, Japan) was applied to the vessel (Figure 3). The course of the patient after endoscopic treatment was uneventful and the patient was discharged 5 d later. Hemoglobin was 108 g/L on the day of discharge. At 6 mo after the endoscopic treatment, there had been no recurrence of upper GI bleeding.

DISCUSSION

Dieulafoy's lesion accounts for 2% to 5% of all cases of acute GI bleeding, but it can be the cause of recurrent and often potentially life-threatening hemorrhage[1-4]. Previous descriptions of the Dieulafoy's lesion emphasized a predilection for the proximal stomach. However, it is increasingly apparent that these lesions, although usually gastric, may develop throughout the GI tract (esophagus 2%, jejunum 2%, colon 10%)[5,6]. A variety of endoscopic modalities, including monopolar and bipolar electrocoagulation, heat probe coagulation, injection sclerotherapy, hemoclip placement, endoscopic band ligation, and laser photocoagulation, have been used, either alone or in combination, with a success rates of 90%-95% and a rate of recurrent bleeding of 5%-10%[7-16]. Because of the small size of the lesion, the

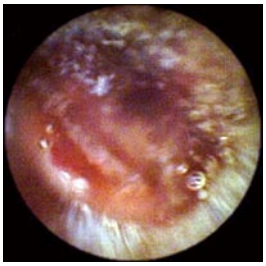


Figure 1 Capsule endoscopy showing a single active bleeding vascular lesion of the small bowel.

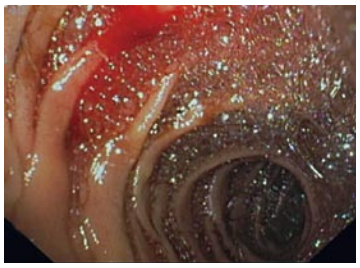


Figure 2 Enteroscopy showing a single oozing vessel with normal surrounding mucosa and no evidence of ulceration.



Figure 3 Application of a hemostatic clip to the vessel.

normal surrounding mucosa and the intermittent nature of the bleeding, endoscopic diagnosis is challenging and may require multiple and combined procedures.

This report describes a case of actively bleeding Dieulafoy's lesion of the small bowel in which the diagnosis was made by capsule endoscopy, followed by treatment with the use of push enteroscopy. Capsule endoscopy is a technically simple procedure that is extremely well tolerated by patients. However, possibility for therapeutic maneuvers cannot be performed with the capsule. By contrast, push enteroscopy is more labor intensive, but it enables various endoscopic therapeutic procedures. Thus, capsule endoscopy is suitable as an initial imaging study for small bowel disorders, whereas enteroscopy is desirable for the confirmation of small-bowel diseases, tissue sampling, and therapeutic applications. The combination of capsule endoscopy and enteroscopy provides a powerful strategy for the diagnosis, as well as treatment of small bowel diseases. As illustrated by the case presented, these procedures are highly complementary.

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CASE REPORT

Recurrence of inflammatory pseudotumor in the distal bile duct: Lessons learned from a single case and reported cases

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Abstract

Inflammatory myofibroblastic tumors (IMTs) or inflammatory pseudotumors (IPs) have been extensively discussed in the literature. They are usually found in the lung and upper respiratory tract. However, reporting of cases involving the biliopancreatic region has increased over recent years. Immunohistochemical study of these lesions limited to the pancreatic head or distal bile duct seems to be compatible with those observed in a new entity called autoimmune pancreatitis, but usually intense fibrotic reaction (zonation) predominates producing a mass. When this condition is limited to the pancreatic head, the common bile duct might be involved by the inflammatory process and jaundice may occur often resembling adenocarcinoma of the pancreas. We have previously reported a case of IMT arising from the bile duct associated with autoimmune pancreatitis which is an extremely rare entity. Four years after Kaush-Whipple resection, radiological examination on routine follow-up revealed a tumor mass, suggesting local recurrence. Ultrasound-guided FNA confirmed our suspicious diagnosis. This present case, as others, suggests that persistent follow-up is necessary in order to prevent irreversible liver damage at this specific location.

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Key words: Inflammatory myofibroblastic tumor; Inflammatory pseudotumor; Local recurrence; Pancreas resection; Whipple procedure

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INTRODUCTION

Histologically, inflammatory myofibroblastic tumor (IMT) is characterized by a proliferation of spindle cells admixed with variable amounts of a lymphoplasmacytic infiltrate. These changes in the pancreatic head produce a mass effect in the biliopancreatic region mimicking a malignant tumor. Thus, surgical resection has been often necessary in order to obtain a definitive diagnosis as well as to relieve symptoms[1]. IMT has been shown to possess potentiality for local infiltration and recurrence with persistent local growth at any organ. Hence, a strict follow-up after surgery is necessary because local recurrence may occur many years later. We report herein a case of local IMT recurrence four years after Kausch-Whipple resection with tumor-free margins in a 55-year-old woman. Primary tumor was located in the distal common bile duct accompanied with lymphoplasmacytic reaction in the pancreatic head. To our best of knowledge, this is the first case reporting a local reappearance of inflammatory pseudotumor in the distal bile duct associated with benign lymphoplasmacytic sclerosing pancreatitis that responded successfully to corticosteroid therapy. Further follow-up in the present case is necessary to elucidate if it behaves as a reactive progressive lesion or tumor.

CASE REPORT

A 55-year-old woman with a past history of IMT affecting the biliopancreatic region [myofibroblastic inflammatory tumor of the distal bile duct (OCD-O code C-24.O M-8825/1) associated with lymphoplasmacytic sclerosing pancreatitis, chronic cholecystitis and reactive lymphadenitis of the isolated nodes] was on routine follow-up after Kaush-Whipple resection.

Four years later, routine surveillance CT scans detected a well defined mass in close relation to the portal vein without apparent infiltration of surrounded structures. At this time, weight loss, malaise or anemia were not present. The tumor mass was well circumscribed, measuring 5 cm × 5 cm, had solid pattern and did not enhance with contrast (Figure 1). An abdominal MRI further delineated that the

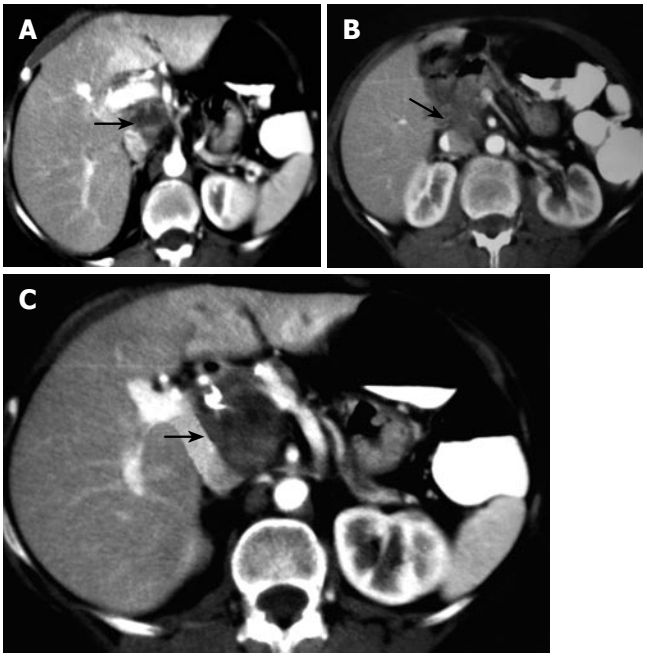


Figure 1 CT scan revealing a well delimited solid mass (arrow), without enlarged regional or para aortic lymph nodes and any evidence of invasion of other organs.

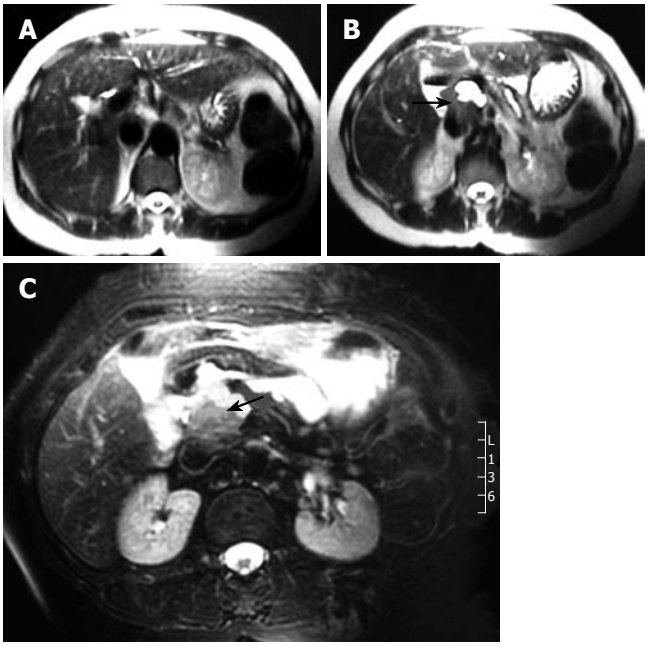


Figure 2 CT scan showing a clear relationship between tumor and inferior cava vein (arrow).

mass was located between the portal vein and inferior vena cava with no signs of widespread infiltration of organs, thereby strongly suggesting the diagnosis of local recurrence (Figure 2). It did not reveal intrahepatic bile duct dilatation but did show chronic changes in pancreatic tissue (Figure 3). Ultrasound-guided fine-needle aspiration (FNA) was performed for cytological examination, which revealed prominent infiltrate of lymphocytes, plasma cells, and acute inflammatory cells without cytologic atypia. These findings were compatible with IMT. Therefore,

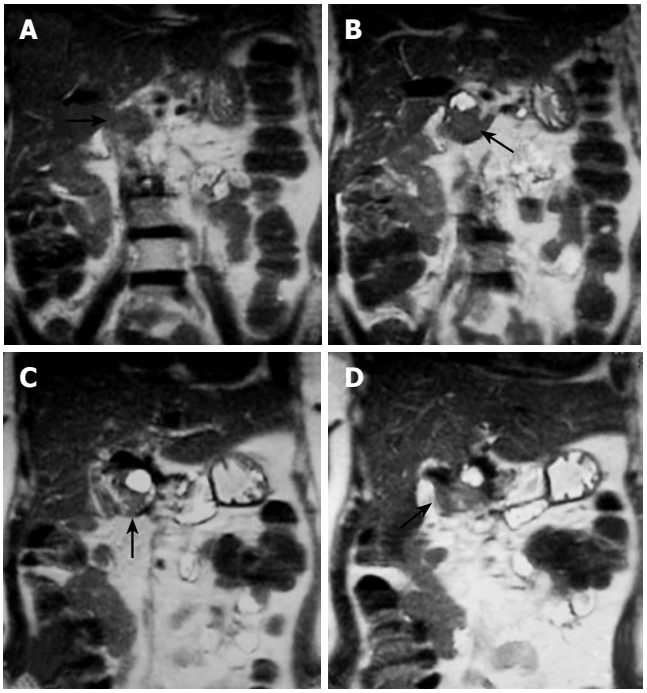


Figure 3 Serial magnetic resonance imaging of the abdomen (coronal plane) showing the tumor mass in the porta hepatis in close relation with the portal vein and biliary tract.

local recurrence showing benign behaviour was suspected. Then, without evidence of systemic disease, corticosteroid therapy was started. A repeat ultrasound revealed complete remission of the tumor at the 3rd mo. The steroid dose is being tapered downward gradually, and we might expect complete remission 6 mo later on low-dose steroid maintenance treatment. On the 5th mo of therapy, the patient remains well and liver function tests were within normal limits.

DISCUSSION

Although IMTs generally behave as benign lesions that are usually cured by radical excision, some cases demonstrate an aggressive growth behaviour. In a study conducted by Coffin *et al*[2], a significant recurrence rate of 25% was demonstrated, likely related to factors precluding complete surgical resection of the pseudotumor. There is a tendency to believe that IMT occurring in the abdomen or retroperitoneum has a propensity for more aggressive behavior with multiple recurrences, invasion into adjacent structures and metastases as shown by DiFiore *et al*[3] and Meis *et al*[4]. However, other recent reports showed that aggressive behaviour, rapid infiltration and multiple recurrences of IMTs are not limited to abdominal location[5-7]. Nowadays, limited evidences exist on the outcomes of patients with a local recurrence of IMT from the biliopancreatic region.

A recent review[1] mentioned reported cases of IMT at the biliopancreatic region, describing locoregional recurrences of four cases at the biliopancreatic region[8,9] after primary resection of the tumor. Further study of these cases showed that just only one of the four cases represented a true local recurrence of pseudotumor arising

from the bile duct. The other three cases were much different from ours because primary tumor mass was located in the pancreas or because the recurrence was found at a distant site (lung) or the recurrence occurred after a long time interval (17 years) from resection and at a different site from primary tumor. The case reported by Johnson *et al*[9] in a 29-year-old black woman recurred after Kaush-Whipple procedure 8 mo later at original site, although the tumor mass was primarily located in the pancreas tissue. Walsh *et al*[8] reported three cases of IMT affecting the biliopancreatic region with long-term follow-up. In one of them, recurrence was found at a distant site (lung). In other case, there was a long interval from resection to recurrence in the pancreatic tail, reason why is not considered a true recurrence. If these recurrences represent a second tumor, multicentricity or metastasis is unknown. In the third case, the tumor recurred at the original site (right main hepatic duct) 14 years later, extending into the liver and showed a benign behaviour. This latter case requires further attention as it is similar to ours but has some interesting differences.

It is widely believed today that IMT at the biliopancreatic region, specially distal bile duct, are usually associated with a new entity called autoimmune pancreatitis which is believed to be an initial activation process. There are clear evidences in literature that most of the reported cases of IMT from the distal bile duct showed association with sclerosing lymphoplasmacytic pancreatitis or autoimmune pancreatitis[1,10-13] and indicates that the fibroblastic cells in both lesions may be induced at the same site and are usually the same inflammatory and fibrosing process with different histological appearances. However, Walsh *et al*[8] did not mention whether the primary tumor at the common bile duct was associated with any form of pancreatitis. Pancreatic tissue was not studied, even they did not mention whether the distal margin was free of inflammatory process. Whether this case represents a form of cholangitis associated with lymphoplasmacytic sclerosing pancreatitis is unknown.

Thus, the case reported by Walsh *et al*[8] that did not show any association with autoimmune process is different from ours in which the distal bile duct and pancreatic head was involved by the same inflammatory process. Other important point is that final post-mortem study of the specimen from the case of Walsh *et al*[8] showed involvement of the liver which suggests and reinforces previous theories that there is possibly an idiopathic pancreatobiliary inflammatory disease complex involving various processes such as autoimmune pancreatitis, although this was not demonstrated.

Apart from this reported cases, our case represent a true local recurrence of pseudotumor of the distal bile duct associated with autoimmune process in the pancreas head. The present case confirms that IMTs have a tendency to recur with time even after radical excision of primary tumor with free margins. These findings support the theory that IMTs are considered as a systemic disease with different pattern of behaviour in which persistent autoimmune reaction process and multicentricity might be involved[13,14].

Our case seem to accord with the theories previously developed by Kawaguchi[15], Zen[13,16] and Klöppel[17] which associates autoimmune pancreatitis with variable involvement of the pancreas and the biliary tract. Fibrosis and zonation[2] may occur as a consequence of persitent inflammatory autoimmune process and biliary stenosis might developed in two different ways: due to external compression from fibrotic changes in the pancreatic head and secondary to zonation of the inflammatory process in the biliary tract which represents our case.

This report provides many important messages. First, local recurrence of IMT at biliopancreatic region has been rarely described. To our knowledge, no cases have been reported in the modern era, which usually associate pseudotumor formation in the distal bile duct with a new entity called autoimmune pancreatitis representing zonation of the same inflammatory process. Second, the biologic potential of inflammatory pseudotumors is highly variable (depending on its location and likely related to factors precluding complete surgical resection, such as adherence to vital structures and multifocality), but it generally has an innocuous course, with more local recurrences than distant metastasis as in our case.

Recurrence is common and the use of corticosteroids is permitted. In these cases, as shown by Voss *et al*[18] and our present case, local recurrences and systemic disease might not require surgery. However, surgery cannot be excluded and long-term follow-up is necessary in order to prevent irreversible liver damage in this specific location. Usually, acute lesions typically respond to high doses of corticosteroid[10], but chronic lesions such as recurrences, which tend to have more fibrosis, might not respond to medical therapy. Third, our case seems to reinforce the hypothesis that inflammatory myofibroblastic tumors from the bile duct might represent zonation by proximity of an autoimmune process affecting the pancreas. This might be explained by pattern of recurrence: our patient presented 4 years ago with indolent jaundice accompanied by biliary dilatation by inflammatory pseudotumor of the distal bile duct. She underwent Whipple procedure with tumor-free margins and negative isolated nodes. However, 4 years later, remaining asymptomatic, radiologic examinations revealed a tumor mass close to the primary lesion without bile duct compression. Moreover, MRI, liver function test and bilirrubin levels were normal. As Whipple procedure separates bile duct from pancreas tissue by interposed bowel loop, where did this recurrence come from? What does the recurrence signify? Perhaps this might be explained by new pseudotumor formation mediated by autoimmune process against remaining pancreatic tissue. Obviously, multicentricity or a new second tumor cannot be excluded.

Many questions still arise about the increasing appearance of inflammatory pseudotumors at different sites: What does really represent inflammatory myofibroblastic tumor? High concentrations of serum Ig subtype, such as IgG4, in IMTs suggest that immunoglobulins may have a pathological role in the fibrosing process mediated by an autoimmune process. Broad pattern of presentation and its ample relation with other autoimmune diseases reinforce this theory[13,16,19-21].

Among other hypothesis, this theory is being increas-

ingly recognized and reflects that inflammatory pseudotumors might represent an advanced stage of an autoimmune process in which intense fibrotic reaction (zonation) predominates[2,17]. What evidences exist in favour of the theory that IMTs at the biliopancreatic region are secondary to autoimmune process in the pancreas? Possible evidences are the frequent occurrence of various autoimmune antibodies, such as antibodies against carboanhydrase II and nuclear antigens[22], the elevated IgG4 serum levels and IgG4 positive plasma cells[13,16,20,23], the oligoclonal pattern of T cell receptor gamma gene rearrangements[24], its association with reactive hyperplastic lymphadenopathy[1,8,25,26], and the responsiveness to steroid therapy of the reported cases[10,27-29]. More importantly, immunohistochemical results of these tumors are compatible with those of other autoimmune diseases. Is it a systemic disease when found at the biliopancreatic region whose course is determined by its biological activity? Strong association between these tumors and other autoimmune processes suggest that IMTs are advanced reactive lesions secondary to an unidentified agent. High concentrations of serum IgG4 found in reported cases at various sites supports that IMTs represent a systemic condition. Hence, it may be localized, multicentric or systemic at the time of presentation and has a strong tendency to reccur. When found at the biliopancreatic region it may represent more than just a biliopancreatic disease.

Some evidences exist about its different biological behaviours that may represent a premalignant state[2,4,5,29-31]. What is the pattern of behaviour of inflammatory pseudotumors at the biliopancreatic region? Limited evidence exists to respond this question, but usually behaves as a benign lesion as reported in the literature to date. Our case represents a local recurrence or multicentricity of IMT at the biliopancreatic region that responds well to conservative treatment. A series of four patients previously described by Walsh *et al*[8] and Voss *et al*[18] demonstrated that IMT from the biliopancreatic region showed a tendency to progressive involvement of multiple organ systems by the same histopathologic process at follow-up.

Is it possible to diagnose preoperatively an inflammatory myofibroblastic tumor presenting with jaundice at the biliopancreatic region and then avoid a complex surgical procedure? A high index of suspicion is necessary to diagnose preoperatively this kind of tumor and exclude malignancy. Medical past history relating other autoinmmune process or previously diagnosed inflammatory pseudotumors at any site should be investigated. FNA with inmmunohistochemical study, and a good response to steroid therapy is necessary to avoid a surgical procedure. Distinguishing inflammatory pseudotumors from malignancy is extremely difficult to do on the basis of radiographic, ultrasound and MRI findings[1,33], specially at the biliopancreatic region as shown by Venkataraman *et al*[34]. Agrons *et al*[35] agreed with them but referring to extra-abdominal sites (lung). The heightened awareness should prompt more extensive non-invasive and minimally invasive radiologic, histopathologic, and inmmunohistochemical evaluations before directing patients toward complicated and potentially high-risk surgical procedures.

Further investigation will improve our understanding

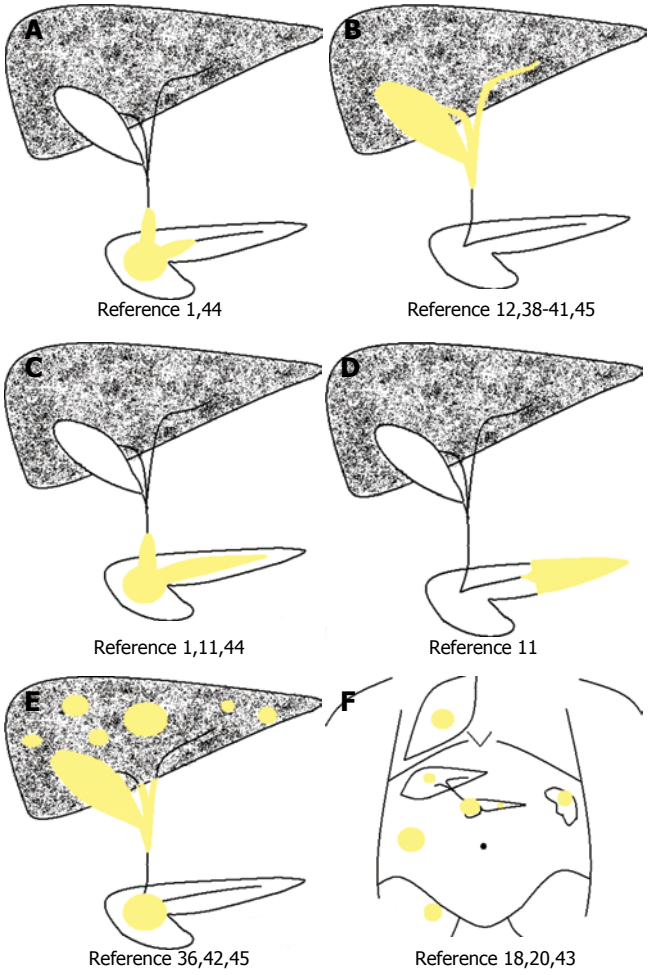


Figure 4 Dynamic classifications for autoimmune pancreatitis. **A-E**: Classification of the variable involvement of the hepatobiliopancreatic system which may be observed in patients with autoimmune pancreatitis. In yellow color is shown the affected zone. [Ref] References of the most relevant and reliable studies that identify this specific pattern of disease. **F**: Multisystemic organ involvement by inflammatory pseudotumor at the biliopancreatic region. In yellow color is shown the affected zone. [Ref] References of the most relevant and reliable studies that identify this specific pattern of disease.

of pathophysiology and etiology of these lesions and such data will help us better recognize this entity preoperatively and differentiate it from carcinomas in order to avoid a surgical procedure. A well illustrated case was the one described by Sasahira *et al*[36].

How should we follow-up these patients? Most organ systems may be involved by primary inflammatory pseudotumors; but also progressive multisystemic organ at follow-up has been described (Figure 4F)[18]. It also has been demonstrated that these chronic process may represent an indolent premalignant state[5,30,31]. Therefore, it is important to follow-up these patients to rapidly diagnose local or multisystemic recurrences in order to avoid complications specifically at the the biliopancreatic region where these compressing lesions might cause liver damage, cholangitis or pancreatitis. Follow-up must not be limited to the biliopancreatic region but also to other systemic organs. Increase rate in specific IgG4 might indicate reappearance of the autoimmune disease and become a useful marker to detect pseudotumor recurrence[37], but this issue remains

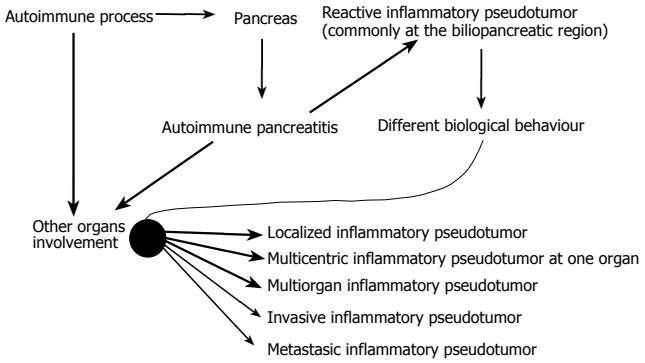


Figure 5 Autoimmune pancreatitis process.

unresolved and controversial[36].

The recurrence in our case is, in fact, represented by multicentricity of the primary tumor, a local recurrence or even systemic disease mediated by an autoimmune process. Overall, we must emphasize that such lesions respond well to corticosteroid therapy and high index of suspicion avoids a surgical procedure.

In conclusion, it seems to be only a question of time until inflammatory pseudotumor will reappear at any site once diagnosed. These lesions most likely will not appear until the etiology is more clearly defined and thus further studies should make a greater emphasis on etiologic factors rather than its presentation form, as virtually any organ may be involved.

Steroid therapy should be the treatment of choice in recurrent cases as good responses have been found in the present and other cases. Follow-up is necessary to avoid complications from recurrences at the biliopancreatic region, especially to avoid liver damage as interval from silent cirrhosis to the development of complication may vary widely. We proposed a new dynamic classification for autoimmune pancreatic-related IMTs[17], with special attention at the biliopancreatic region (Figure 4)[1,11,12,18,20,36,38-44]. Among other theories[45-51], the most widely accepted theory today is that IMT is an auto-immune disorder (Figure 5). IgG4 can be useful as a serological marker of autoimmune pancreatitis and seems also to be useful for the follow-up of patients treated with steroids.

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CASE REPORT

Neuroendocrine gastric carcinoma expressing somatostatin: A highly malignant, rare tumor

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Abstract

Poorly differentiated gastric neuroendocrine carcinomas, although rare, deserve particular attention, as they are aggressive and have an extremely poor prognosis. In this report we describe a gastric neuroendocrine carcinoma with rapidly fatal outcome. Immunohistological staining of the resected specimens revealed that the tumor was an endocrine carcinoma. The tumor disclosed intense immunoreactivity to pan-neuroendocrine markers and diffuse somatostatin immunoreactivity. There were no psammoma bodies and no demonstrable association with von Recklinghausen's neurofibromatosis. In the gastrointestinal tract, neuroendocrine tumors producing predominantly somatostatin have been described only in the duodenum. To the best of our knowledge, the present report is the second case report of a neuroendocrine gastric carcinoma expressing diffusely somatostatin as the only neuroendocrine regulatory peptide.

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Key words: Neuroendocrine carcinoma; Neuroendocrine tumors; Carcinoid; Stomach neoplasms; Somatostatin; Immunohistochemistry; Tumor markers; Enterochromaffin-like cells

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INTRODUCTION

Neuroendocrine [NE] carcinoma of the stomach is a rare, highly malignant tumor with an extremely poor prognosis[1-6].

A subset of NE carcinoma is associated with inherited syndromes, such as multiple endocrine neoplasia [MEN1] and von Hippel-Lindau syndrome[7]. However, the vast majority of NE carcinomas occur sporadically[2,8,9,10].

Poorly differentiated NE carcinomas of the stomach are a separated entity in respect to well-differentiated carcinoids. There is little evidence to support the origin of neuroendocrine carcinomas from malignant evolution of well-differentiated tumors. Rather, it is conceivable that they derive from endocrine-committed immature stem cells, as suggested by the studies of mixed exocrine-endocrine tumors[11,12]. Like type III sporadic carcinoids, NE gastric carcinomas develop independently from hypergastrinemia, thus recognizing a substantially different pathogenetic feature with respect to type I and II enterochromaffin-like (ECL) cell tumors[4,9,13].

The first report of a NE carcinoma of the stomach with diffuse somatostatin immunoreactivity was done by Chejfec *et al*[14]. They reported a neuroendocrine gastric tumor with a rapid fatal outcome, displaying neurosecretory granules by electron microscopy and immunoreactivity to pan-neurosecretory markers, i.e., chromogranin and neuron-specific enolase. The only neuroendocrine regulatory peptide detected in the tumor was somatostatin, identified by immunohistochemistry in the majority of neoplastic cells. In contrast with duodenal somatostatinomas, there were no psammoma bodies and no demonstrable association with von Recklinghausen's neurofibromatosis. Rindi[4] described two gastric neuroendocrine carcinomas in which immunohistochemical staining revealed that 5% to 30% of tumor cells were positive for somatostatin[4]. Yu *et al*[3] reported a case of NE gastric carcinoma expressing somatostatin, but they did not describe the intensity of the staining. To the best of our knowledge, the present report is the second case report of NE gastric carcinoma expressing somatostatin in the majority of the neoplastic cells.

METHODS

Paraffin-embedded specimens containing a representative sample of the tumor were sliced into 3 μm thick sections and mounted on silanized slides. These underwent immunohistochemical analysis using avidin-biotin immunoperoxidase complex technique.

First, the tissue was deparaffinized and hydrated in alcohol. The next step was antigen recovery in a dampness cooker with a steam solution of 10 mmol/L citrate pH 6.0 at 95-100℃ for 35 minutes. Blockage of the endogenous peroxidases was done with a hydrogen-peroxide wash 10V (3%), and following that, non-specific sites were bound with skim milk 2%. Then, the primary antibodies (Dako A/S®, Copenhagen, Denmark) were diluted in this proportion: 1:50 for serotonin, 1:200 for somatostatin, 1:1000 for specific neuronal enolase, 1:800 for synaptophysin, 1:1000 for chromogranin, 1:320 for cytokeratins AE1/AE3, 1:100 for insulin, 1:50 for glucagons and 1:200 for gastrin, in bovine serum albumin (Sigma Diagnostics®, St. Louis, MO). 100 μL of the resulting solution was poured onto the tissue sections and incubated for 18 hours at 4℃.

The slides were washed in sodium phosphate buffer 0.05 M, pH 7.2-7.4 and incubated with the secondary antibody (Large Streptavidin-Avidin-Biotin–Peroxidase System; Dako A/S®, Copenhagen, Denmark). Following this, the slides were developed with chromogene (3-3'-diaminobenzamidine; Sigma Diagnostics®, St. Louis, MO), and counter-stained with Harris' hematoxylin (Sigma Diagnostics®, St. Louis, MO).

The brownish-color was considered evidence of a positive expression of the respective antibodies in the tumor cells. As a positive and negative control of the reactions, histological cuts of human pancreas were used for serotonin, somatostatin, specific neuronal enolase, chromogranin, insulin and glucagon; human colon for synaptophysin and cytokeratins AE1/AE3; and human stomach for gastrin. Uncolored red blood cells were used as negative internal control.

CASE REPORT

A white man, 73 years old was admitted with a 2-mo history of intense epigastric pain, weight loss of 10 kg, loss of appetite and asthenia. Physical examination revealed only discolored mucosa. Laboratory exams were normal except for a hemoglobin level of 9.8 g%. Gastroduodenoscopy showed gastric lesion in the gastric body, with a Bormann III macroscopic aspect, whilst endoscopic biopsies revealed undifferentiated carcinoma. Pre-operatory tomography showed a mass in the gastric region and hepatomegaly with multiple heterogenous nodules suggesting metastasis. Radiographs of the thorax and colonoscopy was normal. On laparotomy a gastric mass was found measuring 6.5 cm in length, located in the gastric fundus and body invading the body and tail of the pancreas and the spleen. Palliative total gastrectomy, omentectomy, splenectomy and distal pancreatectomy were performed to control the patient's pain. Histology revealed a neuroendocrine carcinoma which was poorly differentiated, having medium-sized, polygonal cells, with trabecular or acinar, cytoplasm

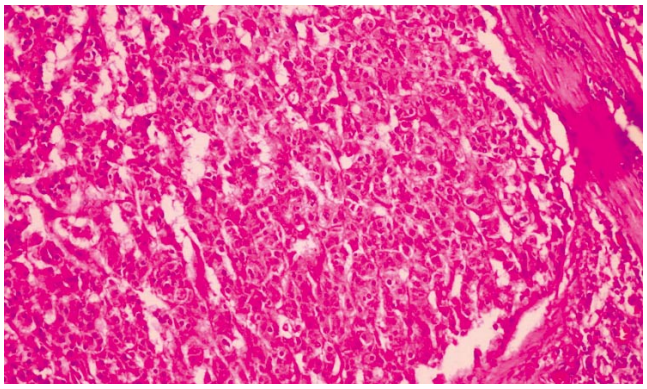


Figure 1 Micrograph of histological section of the neuroendocrine gastric carcinoma showing a proeminent trabecular architecture, organoid pattern with pseudoglandular structure, uniform cells and abundant mitoses. (Hematoxilin & Eosin staining; original magnification, × 200).

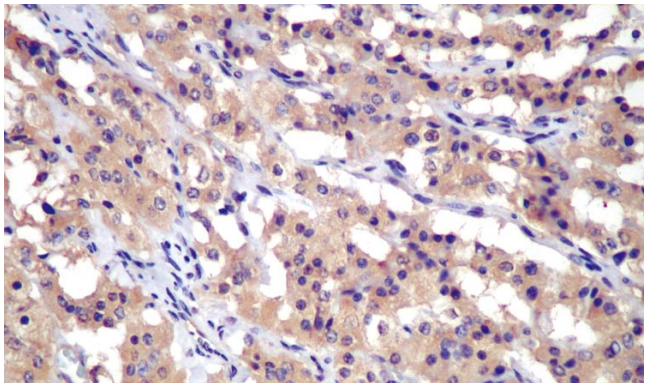


Figure 2 Immunohistochemistry of the neuroendocrine gastric carcinoma showing intense and diffuse somatostatin-positive imunoreactivity in the neoplastic cells (Immunoperoxidase, primary antibody anti-human somatostatin-code A0566-Dako A/S®, Copenhagen, Denmark, original magnification, × 400).

eosinophilic or clear appearance with low pleomorphism (Figure 1). The neoplasia had infiltrated the wall as far as the serosa and there was evidence of vascular and perineural infiltration. The surgical margins were free of any neoplastic infiltration. From a total of 14 perigastric lymph nodes one showed neoplastic involvement. The spleen and pancreas were also infiltrated by neoplasia. Immunohistochemical neoplasia analysis showed immunoexpression for chromogranin, specific neuronal enolase, synaptophysin, cytokeratines AE1/AE3, CAM 5.2 and was strongly positive for somatostatin in the majority of the neoplastic cells (Figure 2). There was no immunoexpression for insulin, glucagon, serotonin, or gastrin. These immunohistochemical investigations of the neoplastic cells confirmed the tumor as a neuroendocrine carcinoma. Post-operatively, abdominal and pelvic tomography showed intra-abdominal collections and the patient was reoperated on the 21st post-operative to drain intracavitary abscesses. An esophageal-jejunal anastomosis fistula appeared and despite parenteral and enteral nutritional support, the patient presented with progressive cachexia and died five months after gastric resection.

DISCUSSION

Gastric neuroendocrine tumor disease embraces a group of tumors that exhibit a spectrum of histopathologic variations ranging from the clearly benign tumors to those characterized by marked cytological atypia and high mitotic activity, which clinically, behave in a highly malignant fashion. In general, the majority of these tumors are well-differentiated, benign ECL tumors, whose courses are indolent and not life-threatening. A relatively small percentage of well-differentiated ECL tumors may have a low-grade malignant potential and deserve an adequate cancer treatment and follow-up. Poorly differentiated gastric neuroendocrine carcinoma tumors are the most aggressive of the group. They have an extremely poor diagnosis, which justifies an aggressive therapeutic approach[4].

Different endocrine cell types have been identified and characterized in the human gastric mucosa[15]. Besides ECL cell producing histamine, enterochromaffin (EC) cell producing serotonin, and G cell producing gastrin, there is the D cell producing somatostatin. Although in theory any one of these endocrine cell types could undergo neoplastic transformation, only the ECL and gastrin [G] cells are found as the exclusive or dominant tumor cell type in oxyntic and antral neuroendocrine tumors, respectively. Conversely, any one or more of the remaining endocrine cell types can be found as minor subpopulation in gastric neuroendocrine tumors[4]. Somatostatin-producing cells were subsequently identified by immunofluorescence in the stomach and in the first portion of the duodenum[16]. Gastric carcinomas display focal somatostatin immunoreactivity infrequently[17] although it is very frequently observed in metaplastic gastric mucosa associated with peptic ulcer or gastric carcinoma[18]. In this present case, none of the peptides generally associated with gastric neuroendocrine tumors were demonstrable by immunohistochemistry except for somatostatin. The diffuse, strong, and very predominant immunoreactivity for somatostatin noted in the majority of neoplastic cells differed from the focal somatostatin reactivity and the coexpression with other hormones reported in neuroendocrine gastric carcinomas[19].

NE gastric carcinomas are in general not associated with an overt endocrine syndrome, even when hormone-like immunoreactivities were detected in tumor tissue, possibly because of the scarce amount of hormone produced or because inactive prohormones, rather than active molecular species, were produced[12,20,21]. Although pancreatic endocrine tumors have commonly been associated with somatostatinoma syndrome, extrapancreatic somatostatinomas are not usually accompanied by a complete scenario of diabetes mellitus, biliary tract disease, diarrhea, steatorrhea, hypochlorhydria, weight loss, or anemia. These gastrointestinal tumors appear to be hormonally inactive and abdominal pain is the main clinical feature[22]. This also occurred with the patient of the present report.

Neuroendocrine tumors of the upper gastrointestinal tract displaying predominantly somatostatin immunoreactivity have thus far only been described in the duodenum[14].

In this case, neoplastic cells expressed immunoreactivity to pan-neurosecretory markers chromogranin A, synaptophysin, and neuron-specific enolase. As in the case described by Chejfec *et al*[14], the predominant neuroendocrine regulatory peptide detected in the tumor was somatostatin, identified by immunohistochemistry in the majority of neoplastic cells. There were no psammoma bodies and no demonstrable association with von Recklinghausen's neurofibromatosis, as is frequently in duodenal somatostatinomas.

NE tumors in non-NE organs are neoplasms that consist of relatively uniform NE cells. These tumors are classified as carcinoid tumors or NE carcinoma based on the combination of NE cell differentiation and cellular atypia. Histologically, NE carcinomas are solid, organoid, trabecular, pseudoglandular, spindle cell, or rosettelike[2]. The tumor of the patient related in this report was composed of malignant neoplasia with cells having a monotome aspect, and exhibiting vascular and perineural invasion as detected in practically all NE carcinomas[21].

Based on both cell size and morphologic features, Matsui *et al*[2] subdivided NE carcinomas into two variants, namely, small and large cell NE carcinoma. Comparing with small cell NE carcinomas, large cell NE carcinomas had a higher mitotic index, larger polygonal cells, a decreased nuclear-cytoplasmic ratio, coarser nuclear chromatin, and more frequently conspicuous nucleoli. Large cell NE carcinoma was an aggressive tumor with a very poor prognosis that approached that small cell NE carcinoma.

There have been many cases of a poorly differentiated adenocarcinoma that show a minor component with histology similar to that of NE carcinoma[1]. It is generally true that NE carcinoma shows the organoid structure of NE tumor despite cellular atypism, but such histological features can be difficult to evaluate in a small biopsy specimen. Thus, it is considered that a diagnosis of NE carcinoma can be made only after histological evaluation of the resected material[23].

In the present patient, the gastric tumor showed a so-called organoid pattern with diffuse immunoreactivity observed for general NE markers. Therefore, the diagnosis of NE carcinoma was considered to be appropriate, although the initial diagnosis based on the biopsy specimen was undifferentiated carcinoma.

The mean age at presentation of these tumors is in the seventh decade, with no significant sex prevalence. NE gastric carcinomas are generally large [mean size 4.2 cm], fungating or annular lesions, found most frequently in the body/fundus. At the time of diagnosis, most of the tumors are already in advanced stage, particularly the lesions with tumor diameter more than 4 cm, and show extensive metastases[2-4,21,24,25]. This clinical picture was also observed in our case.

Although rare, poorly differentiated gastric NE carcinomas deserve particular attention, as they are aggressive and have an extremely poor prognosis. The surgical resection is the most appropriate form of treatment for this type. The usefulness of multi-drug chemotherapy remains to be evaluated in larger clinical studies[2,3,26].

Mean survival rates of 6.5-14.9 mo have been reported with a 1-year survival rate of 58%[3,10,25,26]. As the patient reported in this study, the majority of the patients die due to extensive metastatic disease[1,27].

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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