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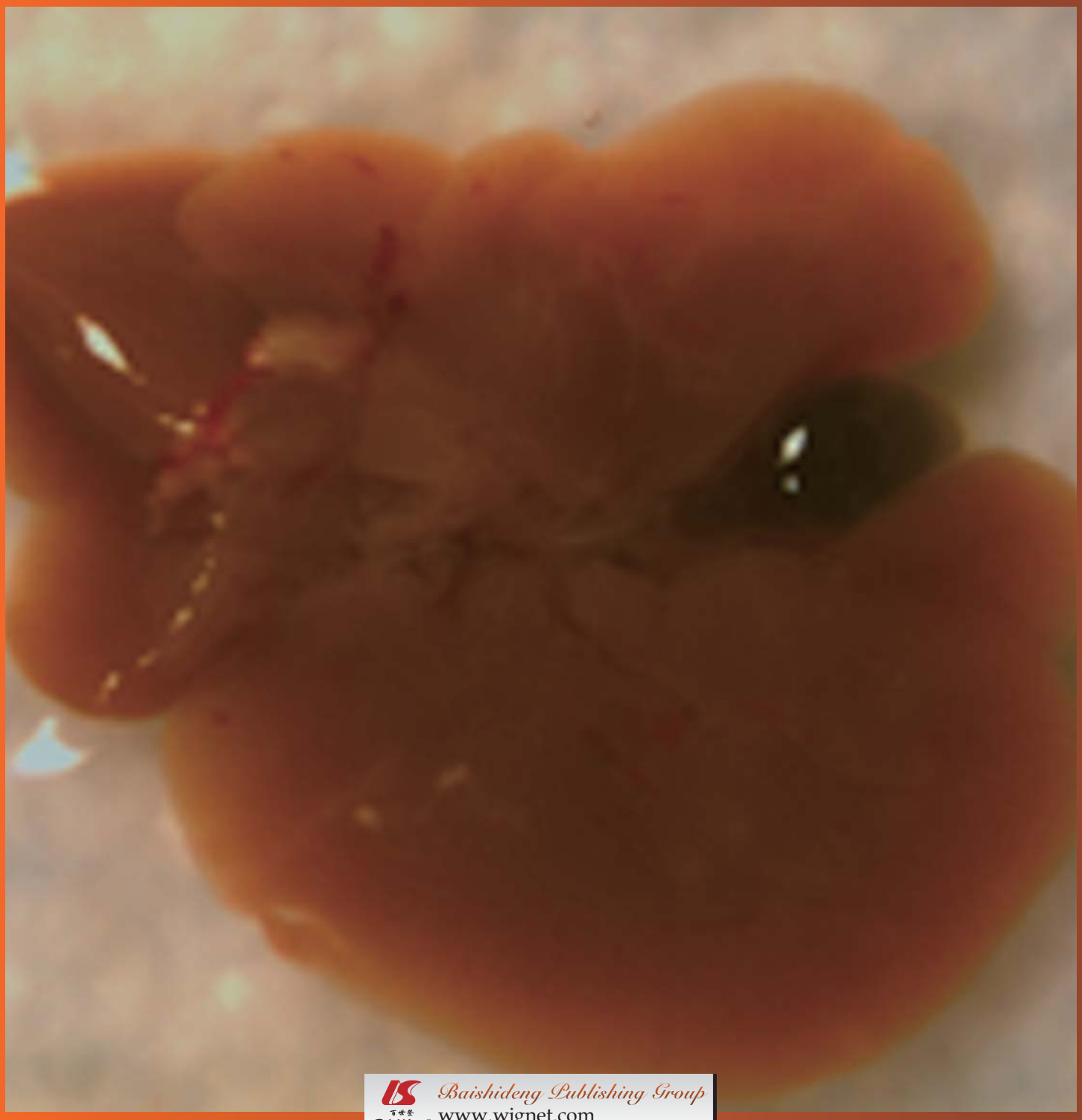

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EDITORIAL

Gastric electrical stimulation for gastroparesis: A goal greatly pursued, but not yet attained

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Abstract

The lack of an effective medical treatment for gastroparesis has pushed the research of new techniques of gastric electrical stimulation (GES) for nearly half a century of experimentation with a large variety of electrical stimuli delivered to the gastric wall of animals and patients with gastroparesis. Three principal methods are currently available: gastric low-frequency/high-energy GES with long pulse stimulation, high-frequency/low-energy GES with short pulse stimulation and neural sequential GES. The first method aims to reset a regular slow wave rhythm, but has variable effects on contractions and requires devices with large and heavy batteries unsuitable for implantation. High-frequency/low-energy GES, although inadequate to restore a normal gastric electro-mechanical activity, improves dyspeptic symptoms, such as nausea and vomiting, giving patients a better quality of life together with a more satisfactory nutritional status and is suitable for implantation. Unfortunately, the numerous clinical studies using this type of GES, with the exception of two, were not controlled and there is a need for definitive verification of the effectiveness of this technique to justify the cost and the risks of this procedure. The last method, which is neural sequential GES, consists of a microprocessor-controlled sequential activation of a series of annular electrodes along the distal two thirds of the stomach and is able to induce propagated contractions causing forceful emptying of the gastric content. The latter method is the most promising, but has been used only in animals and needs to be tested in patients with gastroparesis before it is regarded as a solution for this disease.

Key words: Gastric electrical stimulation; Gastric emptying; Gastric motility; Gastric myoelectric activity; Gastroparesis; Prokinetic drugs

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INTRODUCTION

Gastroparesis is a chronic disorder characterized by a severe functional delay in gastric emptying (GE), which not only causes distressing symptoms, such as upper abdominal discomfort or pain, a sense of epigastric fullness after meals, early satiety, nausea, and vomiting, but may also lead to nutritional depletion requiring enteral or parenteral nutrition. The treatment of this condition represents a clinical challenge and is one of the most disappointing areas in medicine. The current available medical therapy is represented, by dietary modifications and administration of prokinetic agents, such as domperidone, metoclopramide and derivatives, cholinomimetics, such as neostigmine, macrolides, such as clarithromycin, erythromycin, and "motilides" and, more recently, the 5HT$_4$ selective agonists, such as prucalopride[1], while cisapride has been withdrawn from most markets due to its dangerous side effects which

affect the heart. However, some patients with gastroparesis can not undergo chronic treatment with prokinetic drugs, due to the occurrence of severe side-effects, such as nervous disturbances caused by metoclopramide, hyperprolactinemia due to domperidone, and antibiotic activity with erythromycin. In addition, tachyphylaxis may occur with some drugs, such as domperidone and erythromycin, and refractoriness to prokinetic agents is observed in a significant number of patients. The intrapyloric endoscopic injection of botulinum toxin seems to relieve the symptoms of gastroparesis[2,3]. However, a preliminary controlled double-blind study apparently failed to confirm previous results with regard to symptoms, although showed a significant improvement in solid GE[4]. If all of these treatments are unsuccessful and nutrition "per os" is insufficient, patients must undergo enteral nutrition. Surgical jejunostomy performed by laparoscopy and percutaneous endoscopic jejunostomy are indicated for patients with refractory gastroparesis unable to maintain sufficient nutrition "per os", in order to provide nutrients, fluids and medications[5], on condition that there are no motor disturbances of the intestine, such as pseudo-obstruction. If enteral nutrition is not possible, the patient is usually referred to the surgeon for partial or even subtotal gastrectomy with Roux en Y reconstruction[6]. If gastric resection is risky, or refused by the patient, or does not resolve the nutritional problems, the patient must undergo permanent enteral or parenteral nutrition. No alternative to surgery and chronic artificial nutrition was imaginable until 1963, when investigators hoped that gastric electrical stimulation was a new way to cure ileus[7]. Following cardiac stimulation with a pacemaker, these authors thought that it would be sufficient to deliver an electrical stimulus to the gut wall to restore an efficient contraction. However, this simple idea turned out to be more difficult than expected and remained a dream for decades, because the electrical activity, that governs the motor function of the stomach, is much more complex than that of the heart.

The present paper presents a critical overview of various methods of gastric electrical stimulation used in animals and humans aiming to restore efficient gastric motor function and improve dyspeptic symptoms in gastroparesis.

GASTRIC MYOELECTRICAL ACTIVITY

To understand the mechanism of gastric electrical stimulation it is necessary to know what gastric myoelectrical activity is. This consists of an uninterrupted sequence of electrical potential variations called "slow waves", that spring out continuously, at a frequency of about 3/min in man, from a small zone of the proximal gastric corpus near the great curvature (pacemaker area), and propagate distally along the gastric wall toward the pylorus in the form of incomplete depolarization-repolarization annular bands. When the depolarization reaches a determined threshold, the smooth muscle cell membrane depolarizes completely with consequent contraction and another kind of electrical activity, called "spike potentials", appears superimposed on the second part of the slow wave[8,9] (Figure 1). The origin of slow waves lies in the interstitial cells of Cajal type I (ICC), a series of highly ramified cells located between the longitudinal and circular muscle coats, making close contacts with the Auerbach plexus and the smooth muscle cells of both layers mediating the cholinergic excitatory and nitrergic inhibitory inputs[10]. These cells, also called myoneural, have the property of automatically generating and transmitting to smooth muscle cells, the slow waves with an intrinsic frequency decreasing caudally[10,11]. The absence of ICC is associated to the absence of coordinated slow waves[12] and depletion of these cells in pathologic conditions, such as diabetic gastroparesis, may interrupt the propagation of both spontaneous and artificially paced slow waves[13].

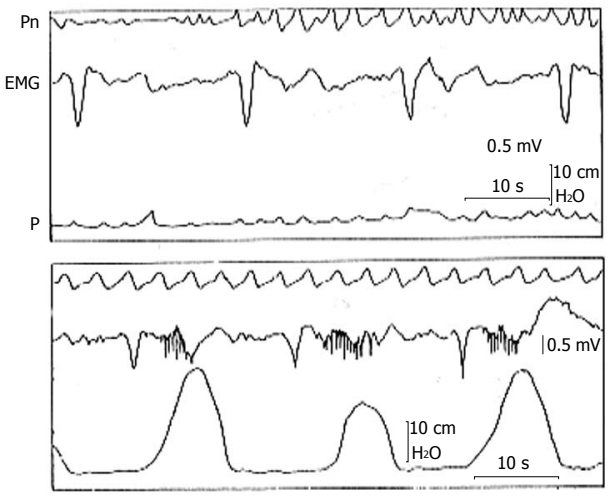

Figure 1 Gastric myoelectric and pressure activities recorded with an intraluminal electromyographic and manometric technique from the gastric antrum of a healthy subject during a period of absence of pressure waves (top tracing) and a period of contractile activity (bottom tracing). Note the slow waves of normal morphology and the frequency of 3 cycles/min that in correspondence with the contractions are followed by bursts of spikes[16]. EMG: Gastric myoelectric; Pn: Pneumogram; P: Pressure.

Hence, the orad area generates the most frequent slow wave activity and functions as a pacemaker. Slow waves initiated at proximal areas migrate caudally and "capture" ("entrain") contiguous distal areas of less frequent intrinsic activity, driving them at their own rate ("coupling"). The slow waves propagate from one cell to another through special contacts in the cell membrane called "nexuses", which provide a pathway of low electrical resistance regulated by the neuro-humoral control system. From these data one can understand the complexity of gastric myoelectrical activity and the importance of its role in gastric motor function. It represents the end point of the motility control system, on which neurocrine, endocrine and paracrine systems operate, and, as it establishes frequency, direction and propagation velocity of peristaltic waves, it may be considered the indispensable condition ("conditio sine qua non") of any coordinated motor activity of the stomach[14].

In gastroparesis, there are more or less severe alterations in gastric myoelectric activity[15], which may be record-

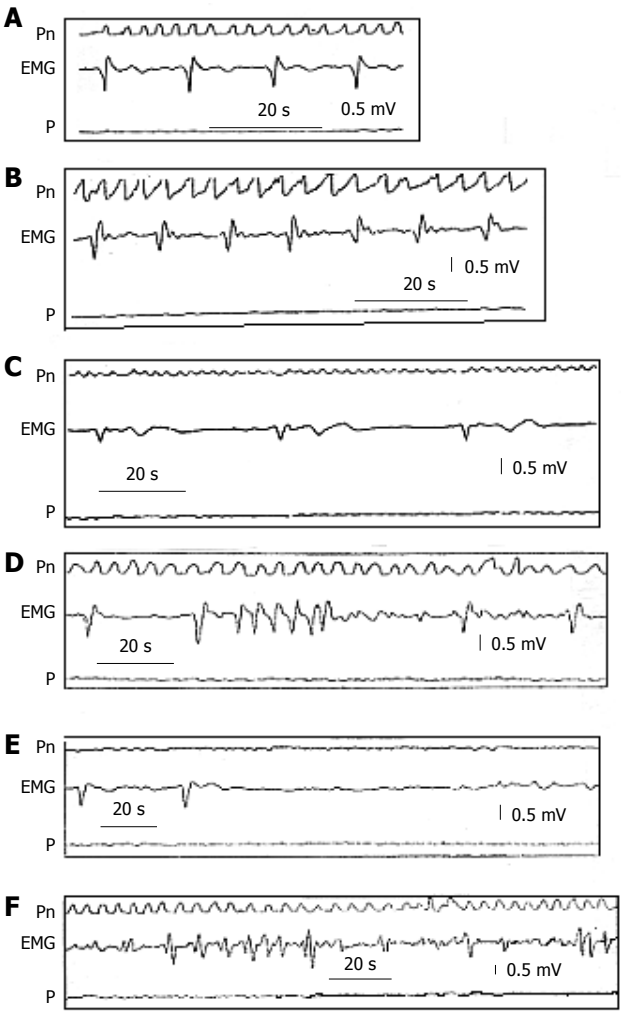

Figure 2 Electrogastrographic alterations. Series of alterations in the gastric myoelectric activity recorded with an electromyographic and manometric technique from the gastric antrum of patients with severe gastroparesis in confront with (A) normal myoelectric activity recorded in a healthy subject showing slow waves of normal morphology and frequency of 3 cycles/min. B: Tachygastria; C: Bradygastria; D: Run of high frequency tachygastria; E: Bradyarrhythmia; F: Complete disorganization of myoelectric activity ("gastric fibrillation"). Note that all these alterations are associated with absence of gastric contractions[1]. EMG: Gastric myoelectric; Pn: Pneumogram; P: Pressure.

ed with intraluminal, serosal and cutaneous electrodes[16,17]. The electrogastrographic alterations consist of various kinds of arrhythmias (Figure 2), very similar to those observed on the electrocardiogram in some cardiac diseases, such as tachygastria, tachyarrhythmia, bradyarrhythmia, asystolia (electrical silence), and gastric fibrillation[16]. The latter is a complete disorganization of gastric electrical activity due to impairment of coupling and propagation of gastric slow waves. All these alterations result in a lack of propagated gastric contractions with a more or less severe delay in GE. However, it is also possible that, despite a regular slow wave rhythm, the gastric wall is unable to contract (electro-mechanical dissociation), because of alterations in the smooth muscle cell contractile system activation and operation.

It is paramount to remember that the motility structures of the gastric wall, such as the smooth muscle cell contractile system, interstitial cells of Cajal pacemaker network, enteric neurons (motor, sensory, integratory) and afferent and efferent fibres connected with the CNS, work using a depolarization-repolarization mechanism. An electrical stimulus delivered to the gastric wall may influence the electrical activity of these structures with consequent modifications of their function and its effect depends on the characteristics of excitability of the target tissues and on the stimulus parameters.

GASTRIC ELECTRICAL STIMULATION

Gastric electrical stimulation (GES) consists of the delivery of electrical stimuli by means of electrodes implanted in the musculature of the gastric wall which are connected to a stimulator device in order to restore effective gastric contractions with normal GE and improve the symptoms of refractory gastroparesis.

Since the 1960s, many investigators have tried to re-establish normal gastric myoelectrical activity to generate coordinated peristaltic activity in patients with refractory gastroparesis[18]. They used a large variety of electrical stimuli differing in pulse width, amplitude and frequency with diverse approaches and various results. It is the difference in frequency of the electrical stimuli from 3-4 cycles/min (cpm) to 50 cycles/s (Hz), which is mainly responsible for the different effects on the target structures of the gut wall.

Two principal types of GES are available: (1) Low-frequency/high-energy GES with long pulse stimulation, the frequency of which is just above that of the native slow wave with a pulse duration in the order of some tenths of a second; and (2) High-frequency/low-energy GES with short pulse stimulation, the frequency of which is markedly above that of the native slow wave with a pulse duration less than one thousandth of a second, delivered singly or in bursts of various length.

Low-frequency/high-energy GES with long pulse (gastric electrical pacing)

The electrical stimulus likely activates the interstitial cells of Cajal and/or muscle cells directly without involving intramural cholinergic nerves, because the administration of atropine does not block the appearance of electrically-induced slow waves[19].

This stimulation is called low-frequency/high-energy GES, because the frequency is slightly above that of the slow wave and its request for energy is high, due to prolonged delivery of a pulse current, for which it is also called "long pulse stimulation". It may be properly defined as "gastric electrical pacing" (GEP), as it refers to electrical stimuli which are aimed to induce propagated slow waves that replace the spontaneous waves.

If a pulse stimulus with a constant current of 2-4 mA lasting 30-500 ms is given to the gastric wall during a non-refractory period, an extra slow wave is induced, which propagates along the gastric wall both in oral and aboral directions, depending on the site of stimulation[20,21]. When a series of stimuli is given, a series of slow waves is induced (entrainment) only if the stimulus frequency is

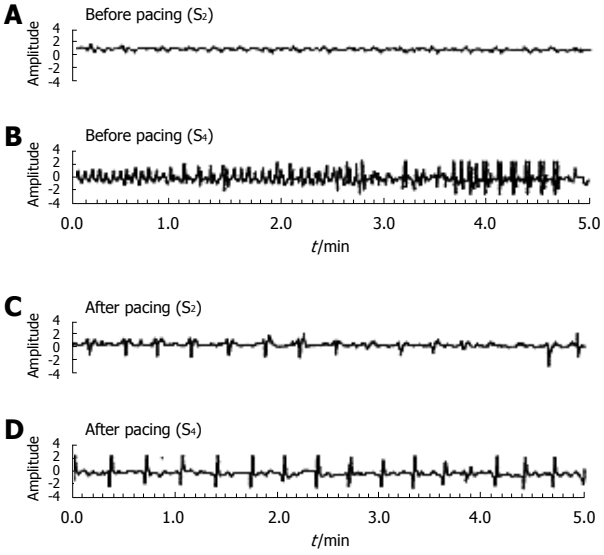

Figure 3 Normalization of ectopic tachygastria with low-frequency/high-energy gastric electrical stimulation [pacing frequency: 3.2 cycles/min (cpm), pulse width: 300 ms, amplitude: 4 mA] in a patient with gastroparesis. The pacing was carried out in the proximal corpus and the recording electrodes were in the mid gastric corpus (S_2) and in the gastric antrum (S_4). Before pacing a slow wave of about 3.5 cpm was recorded at S_2 (A) and tachyarrythmias at S_4 (B), whereas the pacing with a frequency of 3.2 cpm entrained gastric slow waves at S_2 (C) and S_4 (D)[44].

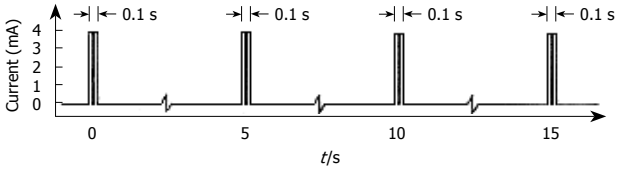

Figure 4 Type of electrical stimulation used by the Enterra system. Short bursts of short duration rectangular pulses (330 μs each) with amplitude of 4 mA were given at a frequency of 14 Hz in each burst. Bursts in turn lasted 0.1 s and were delivered every 5 s[39].

slightly above that of the intrinsic frequency, but not more that 4.7 cpm in man[18,20,22] (Figure 3). The entrainment, however, is not sufficient to re-establish a propagated contraction in all cases and consequently to improve GE, especially if the neuromuscular structures are severely damaged[20]. In fact, GEP in dogs following stimulation of 100-300 ms duration, 5-7 cpm frequency and 2-4 mA amplitude was able to entrain slow waves, even after an artificial gastroparesis induced by the association of vagotomy and glucagon, but the effect on gastric contraction and GE was obtained only in some cases[18,23-27]. However, in patients with gastroparesis, GEP performed by means of an external device and transcutaneous electrodes fixed in the proximal corpus, with a frequency of 3-3.3 cpm, an amplitude of 2-4 mA and a pulse duration of 30-300 ms, was able to induce a regular rhythm in most patients, and in some cases restored efficient contractions, accelerated GE and improved symptoms[28-32].

In conclusion, besides these limitations this kind of extrinsic pacing may re-establish a normal frequency of slow wave activity in gastric dysrhythmias, but does not guarantee the appearance of true contractions and consequent improvement in GE, especially in conditions of severe gastric atony, and has little effect on vomiting. Unfortunately, long duration pulses require high energy which must be provided by batteries too heavy and large to be implanted in a patient for long-term treatment.

High-frequency/low-energy GES with short pulse (gastric neuro-stimulation)

This type of stimulation is called "high-frequency GES" (HF-GES), because the frequency of the stimulation is well above the intrinsic frequency, and is also called "high-frequency/low-energy" GES, because it requires a low quantity of energy, and, being short the length of pulse, is also called "short pulse stimulation".

If a series of stimuli of 2-5 mA amplitude is delivered to the gastric wall with a frequency higher than 4.8 cpm cycles/min at 2-5 mA, no slow waves are induced, because the frequency stimulus is above the "maximum driven frequency" of the stomach. The native slow wave continues to spread with its own frequency and slight modifications, while the effects on contractions and GE are variable[27,33-38]. Stimulation may be performed with a single pulse of constant current[35] of short duration (approximately 300 μs) or by a couple of pulses of 300 μs at 70 μs intervals[34] or by a burst of pulses of high frequency (up to 50 Hz)[33] and variable length. As the power consumption is low, this system does not require unwieldy batteries and allows the implantation of a portable device.

The type of HF-GES most used is performed with an implantable stimulator called Enterra (Medtronics, Minneapolis, MN, USA). It delivers electrical stimuli consisting of couples of pulses with a frequency of 14 Hz, amplitude 5 mA, duration 330 μs, which are delivered for 0.1 s at a frequency of 12 cpm[39] (Figure 4). The electrodes are positioned *via* laparotomy or laparoscopy in the musculature of the gastric corpus, whereas the pulse generator is inserted in a subcutaneous pocket[34,40,41]. This method of stimulation is approved by the USA Food and Drug Administration (FDA) within certain limits on humanitarian grounds and in a few selected centres of research with the approval of the Institutional Preview Board, to be used in patients with diabetic or idiopathic refractory gastroparesis, but has not been authorized by NICE in the UK. Moreover, some centres in the USA have discontinued the implantation of this device, because of few benefits to patients in terms of cost and risks.

With regard to the effects of HF-GES on gastric electro-mechanical activity, the slow waves remain practically unchanged[19,24,26,27,35,37,38], whereas the effects on contractions are contradictory[19,26,27,37]. This may be due to the fact that none of these investigators considered the possible spontaneous occurrence of activity fronts of the migrating motor complex that may increase the motility index casually in correspondence with the period of stimulation. GE was found to be unchanged, worsened or improved, sometimes after months or years of stimulation[26,34,35,38,42-51]. However, these studies were not controlled and in some cases the patients continued to take

prokinetic drugs during the period of stimulation[35,38]. Some investigators solved the problem of little effect on GE by adding a pyloroplasty to GES obtaining an obvious improvement in GE[52]. Other investigators devised a dual stimulation protocol alternating pulses of short duration (0.3 ms) with pulses of long duration (500 ms) every 10 s with the aim of obtaining not only an antiemetic effect, but also to correct dysrhythmias and improve GE[53]. In conclusion, the effect of HF-GES on slow waves and contractions is absent or at least dubious, while there is a slight and inconstant effect on GE.

With regard to the effects of HF-GES on symptoms of gastroparesis, the first uncontrolled trials from a small number of centres reported significant and prolonged gastric symptoms improvement in both diabetic and idiopathic gastroparesis with about 80% reduction in nausea and vomiting[34,46,54-59]. One further study[48] from three regional centres regarding 214 patients carrying the device for an average of 4 years, reported a continued improvement of at least one of the gastroparesis symptoms in 50% to 92% of patients. However, no symptom score and quality of life measurements at baseline and at follow-up were carried out with respect to a control group of 25 non-implanted patients. In addition, no survival benefits were observed in implanted patients with respect to non-implanted patients.

The major fault in all these studies was the absence of a double-blind randomized crossover design, with the exception of one complete study[60] and one abstract[61]. In the first study which included the Enterra system, 33 patients (17 diabetic and 16 idiopathic) were randomized to ON and OFF stimulation for 1 mo periods in a double-blind crossover design, followed by a non-blinded ON period of 6-12 mo. With regard to the results there were, however, as noted in a follow-up "Letter to the Editor"[62], discrepancies between the initial submission to the USA FDA, where a decrease in vomiting frequency was reported without significant differences between the ON and OFF periods, and the subsequent publication in *Gastroenterology*, where a reduction in vomiting frequency was observed in diabetic patients during the ON period and not during the OFF period. The decrease in vomiting frequency continued during the uncontrolled phase of stimulation, confirming the results of other studies[58], but no significant decreases in postprandial fullness, early satiety, pain and bloating were observed. Due to these limited results, the authors announced in a reply to the previously reported "Letter to the Editor"[62], that a new controlled double-blind multicentre trial of GES with Enterra was underway.

The results of a new prospective multicentric double-blind randomized controlled crossover study with Enterra was presented at the DDW of 2010[61] on the effects observed in 32 patients with idiopathic gastroparesis. After 6 wk of stimulation, a double-blind randomized consecutive 3-mo crossover period with the device ON or OFF was followed by an unblinded ON period up to 12 mo after implantation. However, during the crossover period there was a non-significant reduction in weekly vomiting frequency with a median of 9.8 episodes during the OFF period *vs* 6.4 during the ON period. At one year after implantation, symptoms and quality of life were significantly improved as well as GE at 2 h, but not at 4 h.

In patients treated with this system the decrease in vomiting was associated with an improvement in some nutritional parameters, such as body weight and serum albumin, and with a decrease in necessity for parenteral or enteral nutrition. In addition, the need for visits and hospitalization and the use of prokinetic and antiemetic drugs were significantly decreased, whereas in diabetic gastroparesis the glycaemic control was improved, as well as the health-related quality of life[34,46,49,50,56,57,60]. A comparison between medical therapy for gastroparesis and the necessity for health care resources was determined, however, this was carried out only in one study with a very small randomized control group and without a detailed indication of the drugs and doses used[55].

Other studies on the effect of GES were performed in other types of patients with gastroparesis, such as those who underwent a partial gastric resection with or without Roux en Y gastric bypass and those who underwent esophagectomy or heart-lung and kidney-pancreas transplant procedures[42,63,64], who reported a decrease in symptoms for long periods of time. However, the energy requirement for successful stimulation was higher in patients with postsurgical gastroparesis with respect to any other type of gastroparesis[65].

The major problem with this procedure is the scarce responsiveness which may occur in patients with prominent bloating or pain[58], and in idiopathic with respect to diabetic gastroparesis[58,60]. Also, patients with interstitial cell of Cajal loss showed little response to HF-GES[66]. The possible causes of a poor response may lie in the incorrect positioning of electrodes and in opiate use at the time of implantation which may blunt the response to stimulation[58]. Consequently, some investigators suggest an intraoperative endoscopic ultrasound to confirm the correct positioning of the electrodes within the gastric muscle layer[67]. To test the response before the implantation of a permanent stimulatory device, other investigators placed percutaneous stimulating electrodes at the time of gastrostomy or used a PEG technique[68]. Self anchoring percutaneous electrodes have been used[69,70], which may allow prolonged stimulation up to 2 mo[71]. Other investigators propose to adjust the stimulation parameters using hand-held programming devices to increase the voltage or pulse frequency.

Another important problem of the Enterra system is the complications that may take place in up to 20% of patients, such as infections, migration and erosion of the stimulating device[34,60], stomach wall perforation, pain due to adhesive bands from pacing wires to the abdominal wall[72], dislodgment, breakage and erosion of leads into small bowel[73], and stomach wall perforation and intestinal obstruction. All these complications require another surgical intervention and are sometimes lethal[46].

The mechanism of action of this type of HF-GES is unknown. In fact, symptom improvement is not due to an entrainment of slow waves, or to a correction of underlying slow wave dysrhythmias[56,74], or to an improvement in

GE. The improvement in symptoms was associated with a decrease in gastric retention at 4 h in rare cases[43], whereas in the majority of cases there was a discrepancy between the improvement in symptoms and the disappointing results on gastric motor function. A decrease in vomiting with the Enterra system was also observed in patients with hereditary intestinal pseudo-obstruction or with simple functional dyspepsia[70,75], as well as in patients with nausea and vomiting regardless of GE rates[74,76]. These results indicate the existence of a mechanism independent of GE improvement. The fact that in a couple of controlled studies[60,61] a similar improvement in symptoms occurred during the crossover double-blind ON and OFF stimulation, suggests that this mechanism could be a placebo effect or that the surgery itself on the stomach and abdominal wall may have given rise to some kind of afferent stimuli that decreased the sensation of nausea and vomiting. In addition, a spontaneous improvement in gastric motility and dyspeptic symptoms cannot be excluded, as this was noted in patients with postviral gastroparesis and in patients with intractable diabetic and idiopathic gastroparesis under tube feeding, who resumed spontaneously with oral feeding[5,77,78]. No significant effect on the blood levels of gut hormones with gastrokinetic activity, such as motilin, gastrin, neurotensin and pancreatic polypeptide was observed. Other investigators took into consideration modifications in sympatho-vagal activity, adrenergic and cholinergic functions[79,80] or modulation of thoracic spinal neurons activity[81], as well as that of the paraventricular nucleus of the hypothalamus[82] and that of the thalamus, which were found on PET to be activated in gastroparetic patients by gastric stimulation[80]. However, the hypothesis that the device acts through vagal pathways[54,83] was disproved by the fact that HF-GES also works well in patients with vagotomy[42]. Symptom improvement is possibly due to an action on afferents fibres[84] which inhibit the vomiting centre or influence symptom perception in the brain or promote fundic relaxation through nonvagal nitrergic pathways[38,85-87]. In fact, experiments with a gastric barostat showed that HF-GES decreases sensitivity to gastric distension and enhances gastric accommodation to a meal in patients with severe idiopathic gastroparesis[85]. As to why this kind of GES improves nausea and vomiting remains an enigma.

Neural sequential GES is another type of high-frequency GES

The application of a train of electrical square waves with a duration of a few ms and a frequency > 50 Hz with an amplitude of 8-16 V for 4-16 s invokes a contraction of the gut wall at the site of the electrodes. This type of electrical stimulation induces a release of acetylcholine from the intramural cholinergic fibres, which in turn stimulates muscle cell contraction. In fact, its effect is prevented by the previous administration of atropine[88] and for this reason is called "neural GES"[89]. This contraction, however, does not propagate spontaneously, but either circumferentially and aborally[90]. To have a contraction involving a circular band of gastric musculature it is necessary to use

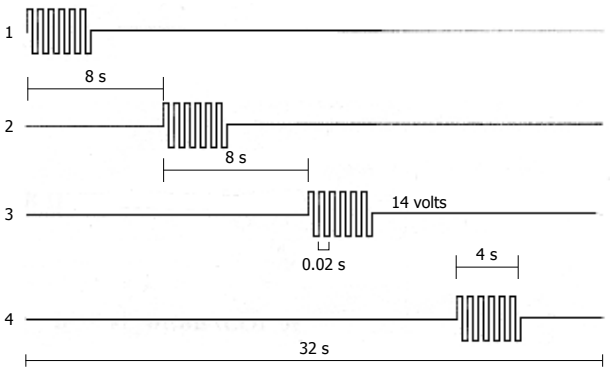

Figure 5 Characteristics of one sequential gastric pacing stimulation protocol in dogs are shown from the proximal (1) to the distal (4) electrodes, that were positioned along the gastric corpus and antrum at 4 cm interval. Four second duration pulse trains with an amplitude of 14 V and a frequency of 50 Hz were delivered in synchronized fashion with a 4 s lag between adjacent stimulus sites[89].

a circular chain of electrodes, and to have a propagated contraction it is necessary to employ a series of these electrodes encircling both the corpus and the antrum, activating them sequentially ("neural sequential GES")[88]. The spontaneous slow wave is overwhelmed by these electronically co-ordinated contractions. This system has the advantage of working both when spontaneous waves show a regular rhythm, but are unable to induce efficient pressure waves, and when slow waves are arrhythmic, uncoupled, completely disorganised and not responding to low-frequency pacing.

Mintchev *et al*[89,91] with the aid of a series of 4-6 ring electrodes placed in the corpus and antrum of dogs, sequentially activated by a microprocessor (Figure 5), was able to induce strong propagated contractions, that increased GE of both liquids and solids. The effectiveness of this type of GES was also demonstrated in a gastroparetic patient at the time of laparotomy[89]. Acute and chronic canine studies confirmed the feasibility of this microprocessor-controlled stimulation method with an implantable multichannel stimulator[92], which may be externally controlled with radiofrequency[93,94].

However, before initiating studies in patients with gastroparesis, chronic experiments in animal models are necessary with an implantable device to evaluate not only the long-term efficiency of this method and the possible incidence of surgical complications, but also to assess the pathophysiologic influence of electrical current pulses on neuromuscular structures of the gastric wall and the effects of the strong antral contractions in the management of gastric content. In fact, one must bear in mind the motor function of the pylorus and duodenum when antral contractions occur. If the pylorus remains open during stimulation, the strong artificial contractions may cause a rapid GE of food particles with consequent risk of maldigestion and dumping syndrome, as suggested by Hasler[95], because the "intestinal brake" is lacking. In addition, if there is a non-propulsive motor disorder of the small intestine, an accumulation of material in the intestinal lumen may take place, which may give rise to a func-

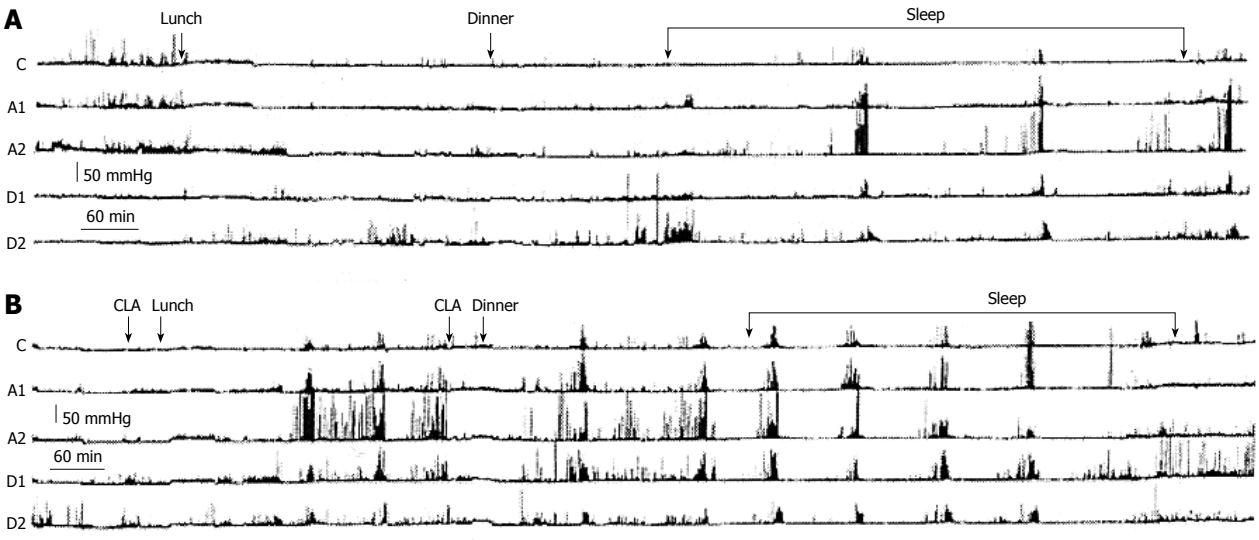

Figure 6 Two ambulatory 24-h gastroduodenal recordings (A and B) carried out on two separate days in a patient with apparently refractory gastroparesis by means of a probe with 5 miniaturized electronic pressure transducers, 5 cm apart: one in the corpus (C), two in the antrum (A1 and A2) and two in the duodenum (D1 and D2). On the first day (A) the recording was carried out without drug administration and on the second day (B) with clarithromycin (CLA) administration. A: On the first day, the postprandial gastric motor activity was very low and only three activity fronts of the Migrating Motor Complex were observed, two during the night and one early in the morning; B: On the second day, the oral administration of clarithromycin 30 min before lunch was followed about 3 h later by a burst of powerful peristaltic contractions starting in the stomach and progressing in the duodenum, followed by two others bursts at about 80 min intervals. The oral administration of clarithromycin 30 min before dinner induced after about 2.4 h a series of six bursts of powerful peristaltic waves in the stomach and duodenum at 80-100 min intervals[103].

tional obstruction. If the pylorus does not open, as may happen in patients with diabetic gastroparesis[96], strong artificial contractions could accumulate the gastric content against the closed pylorus with consequent abnormal antral distension and possible occurrence of pain.

Finally, we are not sure that the normalization of GE will be accompanied by the disappearance of dyspeptic symptoms, if there is visceral hypersensitivity, as happens in patients with dyspepsia despite normal GE[97]. However, we believe that the problem of gastric stasis is crucial in gastroparesis and should be corrected in any case. In fact, besides the severe consequences on symptoms and nutrition and the negative effect on the glycaemic control of diabetes, it may cause "*per se*" gastric damage, such as gastritis (which may be erosive in diabetics), phytobezoars[98] with possible ulceration, obstruction and gastric perforation and pharmacobezoars comprised of medications[99].

From these considerations we believe that an in-depth experimentation of neural sequential GES in various pathophysiological conditions associated with gastroparesis is mandatory.

COMMENT AND CONCLUSION

Many investigators have been engaged in resolving the problem of gastric electrical stimulation in the last half century with different approaches and various outcomes.

Low-frequency/high-energy GES, known as gastric electrical pacing with long pulse stimulation, is able to induce a regular rhythm, restore efficient contractions, and improve GE and symptoms in some cases of gastroparesis, but requires a high quantity of electrical current, which can only be provided by a device too large and heavy to be implanted and for this reason is not suitable for clinical studies. However, with the progress in electronic miniaturization and in the potency of batteries this method may be considered again in the future.

High-frequency/low-energy GES with short pulse stimulation, such as the Enterra system, although it does not significantly modify slow wave and motor activity and does not consistently resolve the problem of delayed GE[34,46,60], shows, however, a good effect on nausea and vomiting with slight but significant improvement in nutritional depletion and health-related quality of life[34,48,57,100]. However, the possibility of spontaneous improvement or a placebo effect cannot be ruled out for sure and there are many considerations regarding the clinical use of this type of GES.

First, one must keep in mind that up to 20% of patients develop more or less severe complications, sometimes lethal, related to implantation of the device[46].

Second, 13% of patients are non-responders[60], especially those with idiopathic gastroparesis, and the improvement in nausea and vomiting may be temporary in 50% of patients[58] and does not include other dyspeptic symptoms, such as epigastric pain, which is an important disabling symptom compelling the patient to continue analgesic therapy[101].

Third, the benefit in nutritional parameters is low, as the average body weight increases were from 0.9 kg[46] to 8.4%[34] a year.

Fourth, when an improvement is obtained, this may not be any better than that obtained by a pyloric injection of botulinum toxin, which avoids the need for surgery in up to 2/3 of patients referred to the surgeon for GES[102]. Moreover, an aggressive drug treatment in suitable doses, taking care to recognize and avoid pseudo-refractoriness to these drugs, may spare implantation of GES. In fact,

in most of the studies examined, including those claiming the superiority of GES over medical therapy[55,56], there was a generic statement of refractoriness to prokinetic and antiemetic drugs without specifying the kind of drugs and dosages. None of the studies considered pseudo-refractoriness due to faulty bioavailability of the drug, which may take place when it is orally administered at the usual time interval of 30 min before meals. We performed a gastroduodenal 24 h-manometric examination in a gastroparetic patient with apparent refractoriness to prokinetics and demonstrated that the drug administered 30 min before a meal took about 3 h to stimulate gastric motility (Figure 6)[103]. Gastric contractions were almost absent during this time, while the meal stagnated in the stomach causing dyspeptic symptoms. One should remember that in these patients about 80% of the gastric content is still in the stomach 2 h after ingestion[60], and that the prokinetic pill also takes this time to reach the intestine to be absorbed and stimulate gastric motility. Therefore, we decided to administer the prokinetic pill more than 2 h before meals to this patient and obtained a marked improvement in dyspeptic symptoms associated with an improvement in GE and a progressive gain in body weight[103].

Fifth, among the considerations that dissuade exposing a patient with intractable gastroparesis to the Enterra system outside of a rigorous placebo-controlled study, there is also the high cost of the procedure which exceeds USD 20 000 and the existence of some limitations, such as the necessity to avoid certain metal detecting security devices and magnetic resonance imaging.

With these considerations in mind, it is advisable to discontinue the use of this type of gastric stimulator for gastroparesis outside properly designed double-blind controlled studies, because it is a costly and risky procedure that does not resolve the principal problem of gastroparesis, that is GE delay, and only improves vomiting without significantly influencing other dyspeptic symptoms, such as epigastric fullness, satiety, anorexia and epigastric pain.

Sequential neural GES, which is able to induce propagated gastric contractions with consequent acceleration of GE, is the most promising method, as it affects the core of the problem of gastroparesis which is gastric stasis, rather than just mitigate the symptoms. However, there is still much research to be carried out, since to date, this method has been used only on animals and one patient with gastroparesis[89,91], therefore it is necessary to use this type of GES in different pathophysiologic conditions of gastroparesis. The hope is that electronic technology could make possible an easily implantable device for humans, able to modulate contractile activity following physiologic necessities. Under these circumstances, GES will become able to treat gastroparesis, whereas to date none of the current technologies has been demonstrated unequivocally to consistently accelerate GE and improve all symptoms in patients with gastroparesis.

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TOPIC HIGHLIGHT

Sang Hoon Ahn, MD, PhD, Professor, *Series Editor*

Quantification of HBsAg: Basic virology for clinical practice

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Abstract

Hepatitis B surface antigen (HBsAg) is produced and secreted through a complex mechanism that is still not fully understood. In clinical fields, HBsAg has long served as a qualitative diagnostic marker for hepatitis B virus infection. Notably, advances have been made in the development of quantitative HBsAg assays, which have allowed viral replication monitoring, and there is an opportunity to make maximal use of quantitative HBsAg to elucidate its role in clinical fields. Yet, it needs to be underscored that a further understanding of HBsAg, not only from clinical point of view but also from a virologic point of view, would enable us to deepen our insights, so that we could more widely expand and apply its utility. It is also important to be familiar with HBsAg variants and their clinical consequences in terms of immune escape mutants, issues resulting from overlap with corresponding mutation in the *P* gene, and detection problems for the HBsAg variants. In this article, we review current concepts and issues on the quantification of HBsAg titers with respect to their biologic nature, method principles, and clinically relevant topics.

Key words: Hepatitis B virus; Hepatitis B surface antigen; Quantitative assay; Virology

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INTRODUCTION

Hepatitis B virus (HBV) causes a wide range of clinical consequences, from acute and chronic infection to cirrhosis and hepatocellular carcinoma, and represents a global public health problem[1,2]. Historically, HBV dates to 1967 when an unknown antigen in Australia was recognized to be associated with hepatitis type B, which was later referred to as the hepatitis B surface antigen (HBsAg)[3]. Since then, HBsAg has served as a qualitative diagnostic marker for HBV infection. Notably, advances have been made in the development of quantitative HBsAg assays, which have allowed viral replication monitoring. A number of clinical studies have evaluated the clinical utility of HBsAg and suggested its potential roles. Yet, it needs to be underscored that a further understanding of HBsAg, not only from a clinical point of view but also from a virologic point of view, would enable us to deepen our insights, so that we could more widely expand and apply its utility. Therefore, in this article, we review current concepts and issues on the quantification of HBsAg titers

(qHBsAg) with respect to their biologic nature, method principles, and clinically relevant topics.

STRUCTURE AND MOLECULAR VIROLOGY OF HBsAg

Components of the viral structure

HBV belongs to *Hepadnaviridae* and is composed of the envelope, core, DNA genome, and viral polymerase. It has a circular form of partially double-stranded DNA and is approximately 3200 nucleotides in length[4,5]. A 42-45 nm long HBV spherical form (Dane particle), which is the full virion with infectivity, can be visualized (Figure 1) under electron microscopy. It has two-layered shells. The outer shell is the envelope protein referred to as hepatitis B surface (HBs) protein, which is further divided into small, middle, and large HBs proteins (SHBs, MHBs and LHBs proteins, respectively), and the inner shell is a core protein referred to as the hepatitis B core protein in which viral polymerase and the HBV genome is enclosed. In addition to the abovementioned full virion, smaller non-infectious subviral particles are present in the serum; 17-25 nm spherical particles, mainly composed of SHBs protein, constitute the most abundant form, which is as much as 10000-fold in excess of the full infectious virion[4,6]. Filamentous (or tubular) particles are another form, with a 20 nm diameter and variable length, and are composed of SHBs, MHBs, and the LHBs protein. The form of the HBV particles appears to be determined by the proportion of LHBs protein[7]. All three forms can be detected in serum with commercial assays and are collectively referred to as HBsAg.

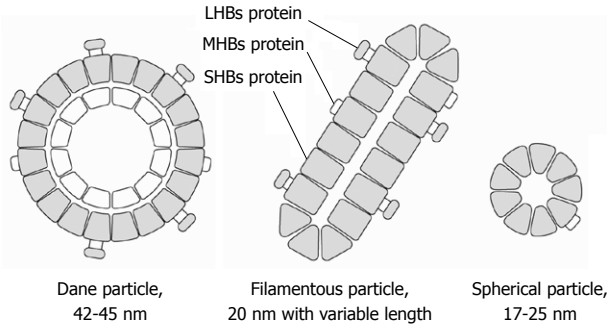

Figure 1 Schematic model of hepatitis B surface antigen structure. Three forms of hepatitis B surface (HBs) antigen (Dane particle, filamentous particle, and spherical particle) are visualized in serum by electron microscopy. These are composed of small, middle, and large hepatitis B surface proteins. LHBs: Large HBs proteins; MHBs: Middle HBs proteins; SHBs: Small HBs proteins.

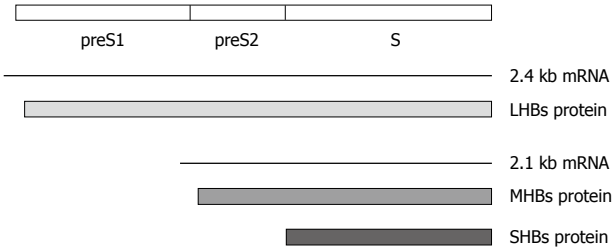

Figure 2 Schematic presentation of the S/preS1/preS2 gene, RNA transcripts, and translational products. Opening reading frame S has three internal AUG codons. Transcription to produce the 2.1 kb and 2.4 kb mRNAs first occurs after translation into small hepatitis B surface proteins (SHBs), middle hepatitis B surface proteins (MHBs), and large hepatitis B surface proteins (LHBs) ensues with different promoters.

Synthesis and secretion

HBV has four distinct open reading frames (ORFs) that encode the envelope, core, polymerase, and X proteins. ORF S has three internal AUG codons encoding the SHBs, MHBs, and LHBs proteins, which correspond to the S, preS2 + S, and preS1 + preS2 + S domains, respectively (Figure 2). These proteins have a common carboxyl end but different amino ends[8].

Like all other proteins, mRNA transcription is the first event to occur. Two 2.1 kb mRNAs for the M/SHBs proteins and a 2.4 kb mRNA for the LHBs protein are formed, and take a separate pathway from viral replication. Diverse transcription factors are involved and act on promoters, enhancers, and other regulatory elements, such as the glucocorticoid responsive element[9,10]. LHBs and M/SHBs expression are thought to be independently regulated with different promoters; a typical TATA box is present in the LHBs promotor (S promotor I , SPI), whereas the TATA-less promotor, which usually has multiple initiation sites, is associated with the M/SHBs promoter, thus accounting for synthesis of distinct proteins from one mRNA. In patients with active viral replication, the protein expression pattern shows a predominance of the M/SHBs protein in contrast to a predominance of the LHBs protein in inactive carriers[11]. After transcription, protein synthesis and glycosylation follows at the endoplasmic reticulum (ER) membrane resulting in a 226 amino acid SHBs protein, the MHBs protein with an additional 55 amino acids, and the LHBs protein with an additional 108-119 amino acids. Although the LHBs mRNA includes the M/SHBs sequence, it does not translate into the M/SHBs protein, and the ratio between the MHBs and SHBs protein is controlled by a complex mechanism, which is not fully understood[12]. To form a full virion, a mixture of HBs proteins in a well-balanced ratio is utilized to envelop core particles in which SHBs and LHBs protein are indispensible[13]. The virion is transported to the cell membrane through vesicles, and several conditions must be satisfied for successful secretion, because excess SHBs protein is required, whereas excess LHBs protein prevents secretion and causes dilatation of the ER with a ground-glass appearance[14-16].

Function

The primary function of the HBs protein as a virologic structure is to enclose the viral components. It also plays a major role in cell membrane attachment to initiate the infection process. Several studies have confirmed the idea that the peptide in the preS1 domain is essential in this process, showing that it specifically binds to the human liver plasma membrane and can be inhibited by a monoclonal antibody[17,18]. However, participation of the SHBs protein in attachment has also been suggested following identification of hepatocyte-bound endonexin Ⅱ, which

specifically binds the SHBs protein[19]. Additionally, from the host perspective, the HBs protein has the major antigenic components, including the *a* determinant, which is important for host-activated immunity. However, from a virologic perspective, it is postulated that excess HBs protein may divert such neutralizing antibody immune function away from the infectious virion[20].

QUANTITATIVE HBsAg ASSAYS

Methods to detect HBsAg were first described in the 1970s using radioimmunoassays and enzyme immunoassays[21,22]. Since then, various diagnostic techniques have been developed, which are mostly confined to qualitatively diagnose HBV in clinical practice. Recently, quantitative assay of HBsAg has been developed, and two commercially available assays will be briefly introduced here.

The Architect HBsAg QT (Abbott Diagnostic, Wiesbaden, Germany) is a chemiluminescent microparticle immunoassay, which is currently the method most widely used in clinical studies[23]. The Architect HBsAg QT assay is a two-step immunoassay with flexible assay protocols, referred to as Chemiflex, for quantitatively determining human serum and plasma HBsAg concentrations. In the first step, the sample and hepatitis B surface antigen antibody (anti-HBs) coated with paramagnetic microparticles are combined. HBsAg present in the sample binds to the anti-HBs coated microparticles. After washing, acridinium-labeled anti-HBs conjugate is added. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of HBsAg in the sample and the RLUs detected by the Architect Immunoassay System optics. The Architect HBsAg is a fully automated system and can detect as low as 0.2 ng/mL of HBsAg with a dynamic range of 0.05-250.0 IU/mL[24].

Elecsys HBsAg Ⅱ (Roche Diagnostics, Indianapolis, IN, USA) is another method for quantitatively determining HBsAg[25]. In the first incubation step, the antigen in the sample reacts with two biotinylated monoclonal HBsAg-specific antibodies and a monoclonal/polyclonal (sheep) HBsAg-specific antibody, labeled with a ruthenium complex, to form a sandwich complex. In the second step, streptavidin-coated microparticles are added, and the complex binds to the solid phase *via* interaction with biotin and streptavidin. The results are reported as a cutoff index (signal sample/cutoff), and the sample is considered reactive if the index is greater than 1.0.

CLINICAL APPLICATION OF QUANTITATIVE HBsAg

Correlation with serum HBV DNA

Although measuring serum HBV DNA is the gold standard for monitoring viral load, it is relatively expensive and not yet readily available in some areas. By contrast, the technique for detecting qHBsAg is fairly easy and inexpensive, and the primary aim of initial clinical studies was to determine the relationship between qHBsAg and serum HBV DNA (Table 1). In 2004, Deguchi *et al*[23] first reported the clinical significance of a high qHBsAg in patients who were hepatitis B e antigen (HBeAg) positive as opposed to those with an antibody positive to the hepatitis B e antigen (anti-HBe), and that qHBsAg correlated well with the serum HBV DNA level ($r = 0.862$). Although there are some contradicting results on whether qHBsAg is correlated with serum HBV DNA[26,27], it seems that they are correlated based on a number of studies[28-33]. Further studies are required to investigate the possibility of using qHBsAg as an aid, if not an alternative, for HBV DNA.

Correlation with covalently closed circular DNA

An important qHBsAg issue is its association with covalently closed circular DNA (cccDNA). cccDNA is a minichromosome and acts as a viral template and replenishing pool for maintaining a chronic HBV infection[34]. Therefore, it is essential to understand the biology of cccDNA when considering HBV therapy. However, to examine cccDNA, an invasive procedure is required, and qHBsAg has been suggested as a surrogate marker for cccDNA. Werle-Lapostolle *et al*[29] reported a significant decrease in cccDNA, qHBsAg, and serum HBV DNA with adefovir (ADV) therapy, and that there was a strong correlation between cccDNA and other variables. This observation was supported by subsequent studies; Wursthorn *et al*[35] and Chan *et al*[36] also showed that cccDNA was significantly correlated with qHBsAg, suggesting that serial monitoring of qHBsAg might act as an additional marker to evaluate treatment response during antiviral therapy.

Prediction of response to antiviral therapy

After the accumulation of data confirming that qHBsAg can be utilized as a viral monitor, qHBsAg has been evaluated as a predictor of virologic response. In a study by Chan *et al*[36] the sensitivity, specificity, and positive and negative predictive values for sustained virologic response (SVR) in patients treated with pegylated interferon (Peg-IFN) + lamivudine (LAM) were 86%, 56%, 43%, and 92%, respectively, with baseline qHBsAg concentrations less than 10000 IU/mL. According to the data of Manesis *et al*[31] achieving the complete elimination of HBsAg would probably require 10.6 years of effective LAM therapy or 5.4 years of a sustained response to interferon. Recently, the clinical usefulness of on-treatment qHBsAg in patients treated with Peg-IFN ± LAM has been suggested in both HBeAg positive and negative patients; a decline in qHBsAg of > 1 log IU/mL or specifically 0.5 and 1.0 log IU/mL at weeks 12 and 24, respectively, had high predictive value for SVR, and on-treatment HBsAg levels could be used as an early predictor of durable off-treatment response to Peg-IFN-based therapy[32,33,37]. Of note is a long-term study by Marcellin *et al*[38] in which 35% of patients who had qHBsAg < 1500 IU/mL at week 12 eventually cleared the HBsAg by 4 years post-treatment, which supports the clinical utility of qHBsAg. Furthermore, qHBsAg was superior to cccDNA and serum HBV DNA for predicting SVR in patients undergoing Peg-IFN-based therapy with receiver

Table 1 Recent clinical studies with quantification of hepatitis B surface antigen titers in hepatitis B virus infection

Author	Antiviral therapy	Correlation	Prediction	Clinical results
Deguchi *et al*[23]	-	HBV DNA	-	qHBsAg is higher in HBeAg(+)
Chen *et al*[28]	-	HBV DNA	-	qHBsAg is higher in HBeAg(+) and high HBV DNA levels, whereas qHBsAg is low in low HBV DNA level CHB
Werle-Lapostolle *et al*[29]	ADV	cccDNA, HBV DNA	-	HBsAg and cccDNA decrease with ADV
Kohmoto *et al*[30]	LAM	HBV DNA	-	qHBsAg is helpful for early detection of drug resistant strains
Wursthorn *et al*[35]	Peg-IFN + ADV	cccDNA	-	Peg-IFN + ADV decreases cccDNA and HBsAg, which are well correlated
Chan *et al*[36]	Peg-IFN + LAM	cccDNA	Low baseline qHBsAg can predict SVR	Peg-IFN + LAM decreases cccDNA and HBsAg, which are well correlated
Manesis *et al*[31]	IFN *vs* LAM	HBV DNA	Low baseline qHBsAg can predict SVR	IFN induces sharper decrease in qHBsAg than LAM
Wiegand *et al*[27]	FAM ± LAM	HBV DNA (not correlated)	Decline of qHBsAg can predict HBsAg loss	2 log drop to below 100 IU/mL is associated with HBsAg clearance
Moucari *et al*[32]	Peg-IFN	HBV DNA	Early qHBsAg drop can predict SVR	qHBsAg may be useful to optimize Peg-IFN therapy
Brunetto *et al*[33]	Peg-IFN ± LAM *vs* LAM	HBV DNA	On-treatment qHBsAg decline can predict sustained HBsAg loss	qHBsAg < 10 IU/mL at week 48 and 1 log decline predict sustained HBsAg clearance to optimize treatment strategy
Lau *et al*[37]	Peg-IFN ± LAM	-	On-treatment qHBsAg can be used as an early predictor of SVR	In HBeAg(+) patients, qHBsAg reduction through weeks 12, 24 and 48 were higher in patients with HBeAg seroconversion
Marcellin *et al*[38]	Peg-IFN ± LAM	-	qHBsAg at week 12 can predict long-term HBsAg clearance	35% of patients who had qHBsAg < 1500 IU/mL at week 12 cleared up HBsAg by 4 yr post-treatment
Lu *et al*[39]	Peg-IFN ± LAM	cccDNA	qHBsAg was superior to cccDNA and serum HBV DNA in predicting SVR	Area under ROC curve with qHBsAg, cccDNA and HBV DNA was 0.769, 0.734, and 0.714, respectively, for predicting SVR
Brunetto *et al*[65]	Peg-IFN ± LAM	-	On-treatment qHBsAg can be used as an early predictor of SVR	On-treatment decline in HBsAg appears to be genotype dependent. Genotype B patients showed the most rapid and pronounced decline
Hou *et al*[66]	Peg-IFN *vs* LAM	-	-	Peg-IFN was superior to ADV in HBeAg seroconversion and qHBsAg decline in LAM-resistant patients

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; qHBsAg: Quantification of HBsAg titers; HBeAg: Hepatitis B e antigen; CHB: chronic hepatitis B; ADV: Adefovir; LAM: Lamivudine; Peg-IFN: Pegylated interferon; cccDNA: Covalently closed circular DNA; FAM: Famciclovir; SVR: Sustained virologic response; ROC: Receiver operating characteristic.

operating characteristic (ROC) curves of 0.769, 0.734, and 0.714, respectively[39].

MOLECULAR HBsAg VARIANTS

Much of our understanding of the biologic nature of the HBs protein has been gathered from various mutation and truncated protein experimental models[40,41], and it is worthwhile to address the relevance and consequences of HBsAg variants from a clinical point of view. Besides the lack of HBV proof-reading capacity[42], the development of an HBsAg mutation can be attributed to immune pressure from extensive vaccination programs, injections of hepatitis B immunoglobulin (HBIG) following liver transplantation, and the overlap with a mutation in the corresponding *P* gene.

Immune escape mutants

Since the introduction of an extensive vaccination program, concerns about HBsAg variants have increased after an HBV infection occurred in infants who had received an HBV vaccination and who had mounted an adequate anti-HBs response. This was presumed to be caused by immune selection pressure, because the HBsAg *a* determinant is the major epitope for HBV vaccination[43,44]. Changes in the amino acids within the *a* determinant, particularly between 137-147, disable surface antigen domain recognition by neutralizing antibodies. Of importance is the G145R mutant, because it is the most common and is replication competent with stability[45]. In a Taiwanese epidemiological study, it was reported that the prevalence of the *a* determinant mutation had increased from 7.8% to 28.1%, after 15 years of a universal vaccination program[46]. Fortunately, in the following years, neither the percentage increase nor any significantly adverse events with an outbreak of HBV infection actually occurred; thus, a mass vaccination program is continuing with adequate justification[47].

In addition to the extensive vaccination program, the wide use of HBIG following liver transplantation adds selection pressure to HBV. Ten of 20 patients who developed recurrent HBV infection despite hepatitis B immunoglobulin prophylaxis had amino acid substitutions involving the *a* determinant, which were mostly absent in pretransplantation clones[48].

Overlap and mutation in the P gene

A mutation in the *P* gene from prolonged oral nucleos(t)ide therapy can cause an altered sequence in the corresponding *S* gene due to overlap of the two genes[49], which is summarized in Table 2[50]. The nucleotide at rt204 in the *P* gene is associated with resistance to LAM, telbivudine (LdT), and entecavir (ETV), and the rtM204V/I mutation typically results in a sI195M, sW196S, sW196L or a termi-

Table 2 Mutations in viral polymerase gene induced by oral antiviral agents and corresponding changes in hepatitis B surface antigen

Polymerase domain	Mutation in polymerase	Oral antiviral agents	Corresponding change in HBsAg
B	rtI169T	ETV	sF161H/L
	rtL180M	LAM, LdT	No change
	rtA181T	ADV, TFV, LAM, LdT	sW172[1]
	rtA181T	ADV, TFV, LAM, LdT	sW172L
	rtA181V	ADV, TFV, LdT	sL173F
	rtT184A	ETV	No change
	rtT184C	ETV	sL175F + sL176V
	rtT184I	ETV	No change
	rtT184G	ETV	sL176V
	rtT184S	ETV	sL175F
	rtT184M	ETV	sL176[1]
	rtT184L	ETV	sL175F
C	rtS202C	ETV	No change/sS193F
	rtS202I	ETV	sV194F/S
	rtS202G	ETV	No change/sS193L
	rtM204V	LAM	sI195M
	rtM204I	LAM, LdT	sW196[1]/S/L
D	rtN236T	ADV, TFV	After end of HBsAg
E	rtM250I	ETV	After end of HBsAg
	rtM250V	ETV	After end of HBsAg

[1]Stop codon. Modified from reference[50]. HBsAg: Hepatitis B surface antigen; ETV: Entecavir; LAM: Lamivudine; LdT: Telbivudine; ADV: Adefovir; TFV: Tenofovir.

nal codon in the overlapping *S* gene[50]. In previous studies, LAM selected HBsAg mutants with reduced anti-HBs binding capacity, and secretion of HBsAg was prevented with a mutant strain due to the stop codon[51,52]. rt181 is another important site that confers resistance to ADV and/or LAM/LdT. Recently, Warner and Locarnini demonstrated that rtA181T caused a secretory defect and had a negative effect on secretion of the wild-type HBV virion because of a concomitant change in the envelope protein at sW172[53]. Similarly, ETV-associated rtI169T/sF161L leads to a decrease in HBsAg immunoreactivity[54].

The clinical significance of overlap and a common mutational substitution in the *S* and *P* gene was further extended by Kamili *et al*[55] who demonstrated a successful experimental infection with the rtV173L, rtL180M, and rtM204V HBV mutants that resulted in sE164D and sI195M despite high anti-HBs levels in chimpanzees[55]. Furthermore, the possibility of a *vice versa* phenomenon with respect to an extensive vaccination program might be postulated in that HBsAg mutants from selection pressure might harbor the corresponding *P* gene mutation, resulting in primary resistance to antiviral agents and therapy failure with these agents.

Detection and variants of HBsAg

As described above, an HBsAg mutation leads to diverse effects, such as decreased secretion and reduced binding capacity to anti-HBs. Of note is that not only a mutation in the *a* determinant but also in the S promoter or a deletion in the preS region can cause such effects[56,57]. These effects may hamper the diagnostic performance of commercial assays, and several reports have pointed to the problem of not being able to detect HBV with an *a* determinant mutation[58,59].

An occult HBV infection is defined as the persistence of the HBV genome in HBsAg negative individuals, and one of the explanations for occult HBV infection is a mutation in HBsAg and undetectability by available assays[60]. Both the Architect HBsAg QT and Elecsys HBsAg Ⅱ seem to reliably detect HBsAg mutants with high sensitivity and specificity[24,25,61]. However, further studies are needed to validate such detection ability, because new or complex combinations of mutations can arise in this era of antiviral agents and extensive vaccination.

FUTURE PERSPECTIVES

Despite progress on qHBsAg, a number of unanswered questions still remain. Precise control mechanisms for HBsAg production in HBV are poorly understood. A discrepancy between qHBsAg and serum HBV DNA exists, although a correlation has been documented. Further research on the virologic nature of HBV could answer these two questions. Meanwhile, the role of qHBsAg in the clinical field is being actively investigated, especially as a predictor to virologic response. Of particular interest is the potential role of qHBsAg for defining the end point of oral antiviral therapy. Current American Association for the Study of Liver Diseases and European Association for the Study of the Liver guidelines with respect to an end point for therapy are unsatisfactory, because reversion to HBeAg positivity does occur after terminating therapy, and the loss of HBsAg is infrequently encountered[62,63]. In this regard, qHBsAg might be particularly helpful in patients with undetectable HBV DNA, even with a highly sensitive polymerase chain reaction assay[64]. In contrast to undetectable HBV DNA, which provides no further information for the virologic responders, HBsAg is continuously shed and detected and, based on the observations of previous studies, qHBsAg with serial monitoring in patients with undetectable HBV DNA may be utilized to determine the end point of therapy and validate the durability of antiviral agents.

CONCLUSION

HBsAg is produced and secreted through a complex mechanism that is still not fully understood. Nevertheless, quantification of serum HBsAg is currently available and there is an opportunity to make maximal use of qHBsAg to elucidate its role in clinical fields. However, a deep understanding of the virology is necessary, and it is also important to be familiar with HBsAg variants and their clinical consequences in terms of immune escape mutants, issues resulting from overlap with corresponding mutation in the *P* gene, and detection problems for the HBsAg variants. Unanswered questions need to be resolved through further qHBsAg research.

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REVIEW

Genomic and genetic alterations influence the progression of gastric cancer

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Abstract

Gastric cancer is one of the leading causes of cancer-related deaths worldwide, although the incidence has gradually decreased in many Western countries. Two main gastric cancer histotypes, intestinal and diffuse, are recognised. Although most of the described genetic alterations have been observed in both types, different genetic pathways have been hypothesized. Genetic and epigenetic events, including 1q loss of heterozygosity (LOH), microsatellite instability and hypermethylation, have mostly been reported in intestinal-type gastric carcinoma and its precursor lesions, whereas 17p LOH, mutation or loss of E-cadherin are more often implicated in the development of diffuse-type gastric cancer. In this review, we summarize the sometimes contradictory findings regarding those markers which influence the progression of gastric adenocarcinoma.

Key words: Gastric cancer; Gene alterations; Prognosis; Molecular pathology

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INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related deaths worldwide, although the incidence has gradually decreased in many Western countries[1]. Several attempts to classify gastric cancer have been made over the past decades. Most successful, and widely used, is the classification by Lauren, which, by microscopic morphology alone, distinguishes two main cancer pathogeneses, diffuse and intestinal subtypes, which clearly appear as dissimilar clinical and epidemiological entities. Although most of the genetic alterations that have been reported are observed in both intestinal and diffuse gastric cancers, it has become apparent that these two tumor types result from different genetic pathways[2] (Table 1).

Microsatellite instability and *p53* mutation, reduced

p27 expression, cyclin E overexpression and 6.0-kb transcripts of the *c-met* gene are involved in malignant transformation from precancerous lesions to intestinal-type gastric cancer. In addition, *DCC* loss, *APC* mutations, 1q loss of heterozygosity (LOH), *p27* loss, reduced expression of tumor growth factor (TGF)-β type I receptor and *HER2* gene amplification are frequently associated with an advanced stage of intestinal-type gastric carcinoma. In contrast, LOH at chromosome 17p (*p53*) and mutation or loss of E-cadherin are more often implicated in the development of diffuse-type gastric cancer, while loss of *p27* and gene amplification of *K-sam* and *c-met* lead to disease progression and metastatic spread.

The two types of gastric carcinoma organize different patterns of interplay between neoplastic and stromal cells through the growth factor/cytokine receptor system, which has a critical role in cell growth, apoptosis, morphogenesis, angiogenesis and metastasis. Other genetic factors, such as DNA polymorphism and genetic instability, may also be implicated in the two distinct major genetic pathways of gastric carcinogenesis.

GENOMIC INSTABILITY

Two phenotypes of genomic instability are generally recognized in gastric cancer: the phenotype associated with microsatellite instability (MSI) and that which is associated with chromosomal instability (CIN). These phenotypes are not necessarily independent and may even overlap in some cases[3].

MSI

MSI is a common feature of gastric cancer due to a deficit in the DNA mismatch repair system and derives from the presence of spontaneous DNA replication errors in simple repetitive sequences[4]. A standard panel of microsatellite markers, including mononucleotide (*BAT26* and *BAT25*) and dinucleotide (*D2S123*, *D5S346* and *D17S250*) repeats, has been recommended and guidelines for MSI testing (Bethesda Guidelines) have been drawn up[5]. Using the reference panel, three levels of MSI can be identified: high-level MSI (MSI-H), low-level MSI (MSI-L) and microsatellite stable (MSS). Recently, it has been established that mononucleotide repeats are instrumental in detecting MSI-H tumors because of their high sensitivity and specificity, and MSI-L has been defined as instability limited to dinucleotide loci[6]. After the adoption of the Bethesda panel, MSI-H phenotype was reported in a range of 5%-50% of all gastric carcinomas with significant differences in various ethnic groups. MSI-H appears to be a phenotypical marker of an underlying cellular defect involving DNA mismatch repair (MMR). Functional inactivation by mutations or epigenetic mechanisms of MMR genes, including *hMLH1* and *hMSH2*, is responsible for the MSI-H phenotype in gastric cancer. Abnormal loss of protein expression of either hMLH1 or hMSH2 has been observed in MSI-H gastric carcinomas[7]. In particular, altered expression of hMLH1 has been associated with gene inactivation by promoter hypermethylation.

MSI-H gastric carcinomas follow a molecular pathway of tumor progression, characterized by the presence of multiple frameshift mutations affecting mononucleotide tracts within genes involved in cancer-related molecular networks which control cellular homeostasis at different levels. MSI-related mutations occur in many genes at variable frequencies[4]. Genes regulating cell-cycle and apoptotic signaling are frequently targeted in MSI-H gastric carcinomas and include *TGFβRII*, *IGFIIR*, *TCF4*, *RIZ*, *BAX*, *CASPASE5*, *FAS*, *BCL10* and *APAF1*[8]. Moreover, genes involved in genomic integrity maintenance, i.e. *hMSH6*, *hMSH3*, *MED1*, *RAD50*, *BLM*, *ATR* and *MRE11*, are also frequently altered in MSI-H tumors[9]. Several studies indicate that, in most MSI-H gastric cancers, multiple target genes are simultaneously mutated and multiple hits impact on different genes in the same pathway[10]. In contrast, gastric carcinomas with MSS and MSI-L exhibit predominant p53 mutations[7].

As compared with MSS or MSI-L, gastric carcinomas with MSI-H show a significantly higher frequency of antral location, intestinal subtype, a lower incidence of lymph node metastasis and improved survival[8,11-15].

CIN

CIN is a feature of various tumors, including gastric cancer, commonly associated with chromosomal aberrations responsible for major modifications of DNA content, i.e. changes in chromosome copy number, and also high-level LOH, gene deletions and/or amplifications[16,17]. All these alterations may lead to oncogene activation and/or tumor suppressor gene inactivation. As with other tumors, aneuploidy is generally considered an unfavorable prognostic factor[18-21], though contrasting results have been reported[22-25].

High CIN levels have also been associated with a shorter survival in gastric cancer patients[26] and high LOH frequencies have been identified at several chromosome arms, including 1p, 3p, 4p, 5q, 7p, 8p, 8q, 9p, 12p, 13q, 17p, 18q, 20q and 22q[27-29].

The allelotype of gastric carcinoma is similar to that of colorectal and esophageal cancers, suggesting the presence of a common genetic pathway for tumor development. Some of these chromosomal segments include genes which are strongly implicated in carcinogenesis, such as the *p53* gene on chromosome 17, *DCC*, *DPC4* and *SMAD2* genes on chromosome 18, and *APC* and *MCC* genes on chromosome 5. Several studies have found that tumors with LOH at chromosome 5q, 18q or 17p had a poorer prognosis than tumors that did not show LOH at these sites[30,31].

EPIGENETIC INSTABILITY

Epigenetic changes, such as aberrant methylation of CpG islands in promoter regions, are commonly detected in human cancers and can permanently inactivate tumor-suppressor genes and affect important pathways of cell cycle regulation and proliferation. The methylation of CpG islands may be considered a third molecular phenotype of

Table 1 Molecular genetic changes in gastric cancer

	Abnormalities	Intestinal phenotype (%)	Diffuse phenotype (%)	Local progression	Distant metastasis	Prognosis	Prolonged survival	Ref.
Microsatellite instability	Mutation, hypermethylation, reduced expression	20-30	0-10	No	NA	Good	Yes	[8,11]
Tyrosine kinases								
HER2/neu	Amplification/overexpression	10-15	0	Yes	Yes	Poor	No	[57-59]
RUNX3	Hemizygous deletion/ hypermethylation/loss of expression	15-45	40-80	Yes	Yes	Poor	No	[62-64]
FHIT	Loss of protein expression (LOH, MSI)	35-65	20-80	Yes	Yes	Poor	No	[65,66]
NM23	Downregulation	3-25	30-70	Yes	Yes	Poor	Discordant results	[73,75]
VEGF	Overexpression	65	35-45	Yes	Yes	Poor	No	[77-79]
HIF-1α	Overexpression	25-60	45-60	Discordant results	NA	NA	Discordant results	[80-82]
COX2	Overexpression	60-70	30-70	Yes	Yes	Discordant results	No	[77,83,84]
SPARC	Overexpression	70-80	25-55	Discordant results	Yes	Poor	No	[85-87]
p53	LOH/mutation/hypermethylation/ overexpression	20-40	20-40	Yes	Yes	No correlation	No	[88-91]
p21	Loss	60		Yes	Yes	Poor	No	[92-94]
p27	Reduced expression	50		Yes	Yes	Poor	No	[95-97]
bcl2	LOH/overexpression	40	0	No	No	Good	Yes	[98]
BAX	Reduced expression	10	5	NA	Yes	Poor	No	[99]
pRb	Reduced expression	60	50	NA	NA	Poor	No	[1,92]
c-myc	Overexpression	45	10	Possible	Possible	Poor	No	[101-104]
	Amplification	15	5	Possible	Possible	Poor	No	
Cyclin E	Amplification/overexpression	15-20		Yes	Yes	Poor	No	[92]
E-cadherin	LOH/mutation/hypermethylation/ reduced expression	0	50	Yes	Yes	Poor	No	[107,108]
MUC1	Overexpression	30-60	15-65	Yes	No	Poor	No	[65,106,110]
PRL-3	Overexpression	30-40	25-60	Yes	Discordant results	Poor	No	[112-114]
Tumor-associated proteases								[115-117]
PAI-1	Overexpression	45-75	35-50	Yes	Yes	Poor	No	
uPAR	Overexpression	40-75	30-50	Yes	NA	Poor	No	
uPA	Overexpression	65	30	Yes	NA	Poor	No	

FHIT: Fragile histidine triad; LOH: Loss of heterozygosity; MSI: Microsatellite instability; VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia inducible factor-1α; COX2: Cyclooxygenase-2; SPARC: Secreted protein acidic and rich in cysteine; PRL-3: Phosphatase regenerating liver 3; PAI-1: Plasminogen activator inhibitor type Ⅰ; uPA: Urokinase-type plasminogen activator; u-PAR: u-PA receptor; NA: Not available.

gastric cancer and the tumor-related genes more commonly methylated are *APC*, *CDH1*, *MHL1*, *CDKN2A*, *CDKN2B* and *RUNX3*. It has also been widely reported that *CDKN2A*, *CDH1* and *MLH1* are more frequently inactivated by promoter methylation rather than by mutations[32].

A series of individual methylated genes has been related to prognosis in gastric cancer. Methylation of tumor-suppressor genes, such as *CDH1*[33], *DKK3*[34], *PTEN*[35] and *MGMT*[36], of putative tumor-suppressor genes, such as *TFPI2*[37] and *CACNA2D3*[38], and of other tumor-related genes, such as *PCDH10*[39] and *SOX2*[40], has been associated with shorter disease-free and/or overall survival.

The combined use of *APC* and *CDH1* methylation markers has identified a subgroup of patients with worse prognosis[41]. Conversely, methylation of single genes has been associated with a better prognosis in some cases. Patients showing methylation of *APC*[42], the M1 region of *MAL* promoter[43] and cyclooxygenase-2 (*COX2*)[44] showed prolonged survival, compared to patients without methylation of these genes.

As with colorectal cancer, the CpG island methylator phenotype (CIMP), characterized by concurrent promoter hypermethylation of multiple genes, has also been described in gastric cancer[45,46] and it has been shown to correlate with hypermethylation of other known cancer-related genes, such as *p16*, *hMLH1* and *THBS-1*[45,47]. Furthermore, the CIMP status is associated with clinically useful information and patients with negative CIMP methylation have significantly shorter survival than those with high CIMP methylation[46,48].

ALTERATIONS OF GENES INVOLVED IN MOLECULAR PATHWAYS

Genetic and genomic variations occurring in genes and molecules that participate in proliferation, invasion and metastasis (e.g. growth factors and their receptors, signal transducers, cell-cycle and apoptosis regulators, cell adhesion molecules, DNA repair genes and matrix metallo-

proteinases) may influence the prognosis of patients with gastric cancer.

Tyrosine kinases

Amplification of some tyrosine kinases (c-met, K-sam and HER2/neu) is associated with human gastric cancer progression. Alternatively, spliced transcripts and enhanced protein expression levels for some of these tyrosine kinases are correlated with the clinical outcome of gastric cancer patients[49].

The oncogene *c-met*, encoding for the hepatocyte growth factor receptor, is preferentially amplified in diffuse-type tumors and has been described to be well correlated with stage and prognosis[50,51]. Overexpression of c-met has also been shown to be associated with lower survival probability[52,53].

K-sam oncogene, a member of the fibroblast growth factor receptor family, is more frequently activated in diffuse-type tumors[2]. Overexpression of K-sam occurs in approximately 32% of diffuse-type gastric cancers, and the prognosis of K-sam-positive patients is poorer than that of K-sam-negative patients[54].

The HER2 protein (HER2/neu or ErbB-2) is a glycoprotein with tyrosine kinase activity, homologous to the epidermal growth factor receptor. HER2 is codified by a gene located on chromosome 17q21 and does not bind to any known ligand. Some studies demonstrated that overexpression of c-erbB2 is selectively found in intestinal tumors and may serve as a prognostic marker for tumor invasion and lymph node metastasis. Overexpression of HER2 protein in gastric cancer has been reported to range from 7.4% to 38%[55-57]. The prognostic value of HER2 expression and/or amplification has been widely investigated with controversial findings. Although most available studies indicate that the overexpression of HER2 is an independent prognostic factor associated with a shorter disease-free[58] and overall survival[57-59], some studies failed to confirm its prognostic role on multivariate analysis[51] or to find a correlation between HER2 overexpression and survival parameters[56,60]. Also associated with poor survival is the presence of HER2 amplification[61].

RUNX3

RUNX3, a gene that codifies for a member of the runt domain-containing family of transcription factors, frequently shows loss of expression due to hemizygous deletion and hypermethylation in gastric cancer. This gene, generally expressed in only 45%-50% of gastric cancer patients[62,63], positively regulates the expression of BIM and p21, and negatively regulates vascular endothelial growth factor (VEGF), thus affecting apoptosis, cell growth arrest and angiogenesis. The loss or substantial decrease of RUNX3 protein expression in gastric cancer has been significantly associated with shorter survival[62,64].

FHIT

The fragile histidine triad gene (*FHIT*) encodes a diadenosine 5',5'''-P1,P3-triphosphate hydrolase and is generally inactivated by deletion or methylation in several tumors, including gastric cancer. The absence of FHIT protein has been shown to correlate with higher tumor stage and histological grade[64], as well as with poor overall survival[65,66].

NM23

The *NM23* gene maps to chromosome 17q21 and encodes the nucleoside diphosphate kinase A, a member of the NDP kinase family. NM23 expression is reduced in metastatic melanoma and breast cancer cell lines[67]. Transfection into cell lines affects invasion, motility, colonization, differentiation and liver metastasis[68]. Decreased expression of NM23-H1, the human homologue, is found in advanced stages of human cancer[69,70].

The expression of the putative metastasis-suppressor gene *NM23* in gastric carcinoma is controversial. In several studies, expression of NM23 has been shown to be inversely correlated with the metastatic potential of gastric cancer[71,72] and with prolonged overall survival[73]. The results of other studies, however, suggest that *NM23* is not a metastasis suppressor gene and does not show correlation with metastasis[74,75].

VEGF

VEGF is a pro-angiogenic factor, frequently overexpressed in tumors. Mutations of *p53*, which under physiological conditions downregulates VEGF, may be responsible for its overexpression[76].

A correlation of the expression of VEGF with lymph node and liver metastasis has been described[77] and patients with VEGF-positive tumors have a rather worse prognosis than those with VEGF-negative tumors[78,79].

HIF-1α

The hypoxia inducible factor, HIF-1α, is a transcription factor that plays an essential role in cellular and systemic homeostatic responses to hypoxia. The prognostic role of HIF-1α expression in gastric cancer patients is controversial: high levels have been associated with a shorter overall survival[80], but also with no difference in survival parameters[81]. However, its upregulation (high HIF-1α mRNA or protein levels) has been found to be positively correlated with VEGF[82] or p53[80] protein expression in gastric cancer patients, and overall survival of patients with high mRNA levels of HIF-1α and VEGF, as well as of HIF-1α and p53, was shorter compared to patients with different features.

COX2

COX2 is one of the key isoenzymes in the production of prostaglandins, and is thought to be involved in carcinogenesis. Some studies indicate that COX2 may play a role in the development of gastric cancer, and its overexpression is associated with nodal metastasis, tumor invasion and differentiation, implicating a poor prognosis[77,83,84].

SPARC

The secreted protein acidic and rich in cysteine (SPARC or osteonectin) is a member of a family of matricellular

proteins that modulates cell-matrix interactions and cell function without participating in the structural scaffolding of the extracellular matrix. Since SPARC alters membrane permeability, cell shape, proliferation, migration and attachment, it may play a role in angiogenesis. It has been reported that its overexpression correlates with distant metastasis and poor prognosis[85-87]. It is not clear whether SPARC overexpression is a useful marker in the prediction of lymph node metastasis development[85].

p53

The p53 protein plays a fundamental role in cell growth and division. The function of the *p53* gene is more frequently altered due to LOH and mutation than to DNA methylation. Mutations of *p53* are present in about 40% of early and advanced, well-differentiated gastric cancers[88]. A lower incidence of *p53* mutations has been shown in young patients compared to older patients[89].

p53 can be investigated by immunohistochemical techniques, bearing in mind that the half-life of the p53 mutant protein is prolonged. Cells carrying the p53 mutant protein can be stained with antibodies against p53, whereas cells carrying normal p53 are negative. Sequencing of the gene after screening can also be performed in order to determine the mutation location within the gene[90].

Overexpression of p53 often occurs in the early stages of intestinal-type tumors, and there is no significant difference between early and advanced cancers. In contrast, p53 abnormalities are not often seen in the early stages of diffuse-type tumors, but tend to occur as the disease progresses[91].

p21

p53 cell cycle regulatory function is mediated by different effectors. One of these is a cyclin-dependant kinase inhibitor (CDK I), the p21 protein. The cell cycle check points are controlled by a cascade of phosphorylation. Protein kinases such as cyclin-dependent kinases are activated by cyclins and inhibited by CDK I, although p21 is up-regulated not only through a p53 pathway, but also through a TGFβRII pathway.

Levels of p21 expression could indicate the absence of a functional p53 protein in neoplastic cells. It has been reported that the survival of gastric cancer patients with p21-positive tumors is significantly longer than that of patients with p21-negative tumors[92]. The expression of p21 is usually assessed in combination with p53 status and contributes to predicting the clinical outcome of gastric cancer patients[93,94].

p27

It has been suggested that the cyclin-dependent inhibitor p27, which controls the transition from G1 to S in the cell cycle, has prognostic relevance in gastric cancer. Reduced p27 expression is detected in approximately 40%-50% of gastric cancers[28]. Some studies have shown that tumors with a low expression of p27 protein are poorly differentiated and at an advanced stage[95,96]. However, some authors have found no difference in overall survival of gastric cancer patients whether with high or low p27 expression[97]. p53, p21 and p27 have also been analyzed in combination, confirming their role as prognostic markers[91].

BCL2

BCL2 and *p53* are closely linked in the regulation of apoptosis. LOH at the *BCL2* locus is frequently observed in gastric cancer. The overexpression of BCL2 may have a role in the development of gastric cancers. It has been shown that BCL2 overexpression reduces cellular proliferative activity and correlates with a less aggressive biological behavior of the tumor. The prognostic role of BCL2 on its own or in association with p53 has not yet been elucidated[98].

BAX

BAX gene encodes a protein belonging to the BCL family members. Negative BAX protein expression has been associated with de-differentiation, lymph node metastasis and shorter survival, suggesting that BAX status may play a role in the development and differentiation of gastric cancer and tumor progression[99].

pRb

pRb encodes a protein that is a negative regulator of the cell cycle. Poor prognosis of gastric cancer patients with low levels of pRb expression has been reported[92,100].

c-myc

c-myc gene encodes a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. The c-myc protein has been shown to be significantly enhanced in well-differentiated gastric cancer[101] and associated with a poor prognosis[102]. Although c-myc is a short-lived protein in normal cells, its stability is increased in transformed cells through several mechanisms. One of these has recently been identified in the overexpression of a human oncoprotein, the cancerous inhibitor of protein phosphate 2A (CIP2A) that stabilizes c-myc[103]. Interestingly, the expression of CIP2A has been associated with reduced overall survival in gastric cancer patients[104].

Cyclin E

Cyclin E overexpression correlates with invasiveness and proliferation and may be a marker of tumor aggressiveness. Although somatic mutations of the cell cycle inhibitor $p16^{MTSI}$ are rare, its reduced expression is associated with depth of invasion and metastatic potential in both diffuse- and intestinal-type gastric carcinomas. However, recent data show that the survival of gastric cancer patients with cyclin E-positive tumors is not significantly shorter than that of negative patients[92].

E-cadherin

Cell adhesion molecules are implicated in human carcinogenesis. Cadherin is a superfamily of calcium-mediated membrane glycoproteins, forming one of the four classes of adhesion molecules. E-cadherin, one of the members

of the trasmembrane glycoprotein family expressed by epithelial tissues, not only acts as a cell adhesion molecule, but also plays an important role in growth development and carcinogenesis. The intact function of E-cadherin is crucial for the establishment and maintenance of epithelial tissue polarity and structural integrity. Around 25%-40% of hereditary diffuse gastric cancers are caused by heterozygous E-cadherin. The inactivation of the second allele occurs by mutation and methylation events, and this results in the complete inactivation of the protein[105]. Reduced expression of E-cadherin correlates with infiltrative and metastatic ability in gastric cancer[33] and the gene encoding E-cadherin, *CDH1*, was among the first to be considered as an invasion suppressor gene. Patients with E-cadherin-positive gastric cancers showed statistically significant prolonged 3- and 5-year survival rates, compared to patients with E-cadherin-negative tumors[33,106].

It has been shown that serum soluble E-cadherin is increased in several non-neoplastic diseases and also in various cancers, including gastric tumors. E-cadherin may be a potentially useful prognostic marker and high levels of soluble E-cadherin correlate with the depth of tumor invasion, as well as inoperability[107]. In addition, levels higher than 10000 ng/mL predict a survival of less than 3 years in more than 90% of patients[108].

The Wnt-frizzled-β-catenin signaling pathway is frequently activated in gastric carcinoma (e.g. upregulation of *Wnt* gene expression or of genes for Wnt ligand receptors, upregulation of *RAC1* and inactivation of *APC*), leading to poor differentiation and increased tumor invasiveness[109].

MUC1

Mucins are high-molecular weight glycoproteins containing oligosaccharides. These glycoproteins constitute the major components of the mucus that protects the gastric epithelium. Overexpression of mucin 1 (MUC1) has been linked to poor prognosis in gastric cancer patients[65,110].

It has been reported that MUC1 may accelerate tumor invasion by the impairment of E-cadherin[111]. The combined expression of MUC1 and E-cadherin shows that survival for gastric cancer patients with abnormal E-cadherin/MUC-positive expression was shorter than for patients with other expression patterns[106].

PRL-3

The phosphatase regenerating liver 3 (*PRL-3*) gene encodes a protein belonging to a class of prenylated protein tyrosine phosphatases. These proteins are cell signaling molecules with a regulatory role in several cellular processes. The prognostic role of PRL-3 in solid tumors, including gastric cancer, has been recently reviewed by Bessette *et al*[112]. High expression of PRL-3 has been associated with several unfavorable clinical parameters, such as tumor size, depth of invasion, lymphatic invasion, advanced stage and shorter overall survival. Successive studies have confirmed these findings[113,114].

Tumor-associated proteases

Tumor-associated proteases and their inhibitors play a central role in tumor invasion and metastasis. The positive correlation of histological data with the urokinase-type plasminogen activator (uPA) and the plasminogen activator inhibitor type I (PAI-1) has been reported. Moreover, the independent prognostic impact of both uPA and PAI-1 on the survival of gastric cancer patients has been demonstrated. Elevated uPA and PAI-1 levels have been shown to be associated with shorter survival[115,116]. A trend towards poor prognosis has also been observed in patients with high expression of the u-PA receptor (u-PAR)[113] and the uPA system may therefore be a target for novel therapeutic agents.

The prognostic role of some uPA genotypes has recently been investigated and an association was demonstrated between the exon 6 C/T polymorphism with invasive phenotype, but not with susceptibility or survival[117].

CONCLUSION

Gastric carcinomas are histologically and genetically heterogeneous and are influenced by gene-environment interactions resulting in the activation of multiple molecular pathways. The molecular subtypes of gastric cancer include three main groups of tumors characterized by either the CIN, the MSI or the CIMP pathways. Currently, it is not clear whether or in what way knowledge of these subtypes of gastric carcinomas is of use in clinical practice, with regard to predicting specific pathways with mutational and regulatory alterations that may interfere with targeted therapies.

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ORIGINAL ARTICLE

Hepatocellular carcinoma xenograft supports HCV replication: A mouse model for evaluating antivirals

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Author contributions: Hazari S performed the majority of the experiments, acquisition and analysis of data, and participated in design of the study; Hefler HJ performed some animal experiments and helped in the antiviral studies; Chandra PK performed the real time RT-PCR and RPA; Poat B and Gunduz F performed biochemical assays and tumor cell inoculation of SCID mice; Ooms T performed the intrasplenic injection of S3-GFP cells into SCID-NOD mice and gave valuable suggestions on animal handling and care during the whole study; Wu T performed the histological evaluation of subcutaneous and liver tumors, and took all the histological images; Balart LA provided valuable suggestions and edited the manuscript; Dash S conceptualized and designed the study, evaluated the overall results, and wrote the manuscript.
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Abstract

AIM: To develop a hepatocellular carcinoma (HCC) xenograft model for studying hepatitis C virus (HCV) replication in a mice, and antiviral treatment.

METHODS: We developed a stable S3-green fluorescence protein (GFP) cell line that replicated the GFP-tagged HCV sub-genomic RNA derived from a highly efficient JFH1 virus. S3-GFP replicon cell line was injected subcutaneously into γ-irradiated SCID mice. We showed that the S3-GFP replicon cell line formed human HCC xenografts in SCID mice. Cells were isolated from subcutaneous tumors and then serially passaged multiple times in SCID mice by culturing in growth medium supplemented with G-418. The mouse-adapted S3-GFP replicon cells were implanted subcutaneously and also into the liver of SCID mice *via* intrasplenic infusion to study the replication of HCV in the HCC xenografts. The tumor model was validated for antiviral testing after intraperitoneal injection of interferon-α (IFN-α).

RESULTS: A highly tumorigenic S3-GFP replicon cell line was developed that formed subcutaneous tumors within 2 wk and diffuse liver metastasis within 4 wk in SCID mice. Replication of HCV in the subcutaneous and liver tumors was confirmed by cell colony assay, detection of the viral RNA by ribonuclease protection assay and real-time quantitative reverse transcription polymerase chain reaction. High-level replication of HCV sub-genomic RNA in the tumor could be visualized by GFP expression using fluorescence microscopy. IFN-α cleared HCV RNA replication in the subcutaneous tumors within 2 wk and 4 wk in the liver tumor model.

CONCLUSION: A non-infectious mouse model allows us to study replication of HCV in subcutaneous and metastatic liver tumors. Clearance of HCV by IFN-α supports use of this model to test other anti-HCV drugs.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Tumor xenograft; SCID mouse; Interferon-α; Antiviral agent; Virus replication

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INTRODUCTION

Hepatitis C virus (HCV) is the most common blood-borne infection that affects the liver. The majority of individuals infected with HCV end up with a chronic disease in which the virus replicates in the liver for a long period of time. There are approximately 170 million people currently infected with HCV worldwide[1,2]. The incidence of new HCV infections each year is increasing in developing nations due to blood transfusion from unscreened donors, which makes HCV a significant worldwide public health problem. The standard treatment option for chronic HCV infection is combination of pegylated interferon-α (IFN-α) with ribavirin[3]. However, the majority of chronic HCV patients do not clear the virus infection with this treatment, and these individuals remain at high risk for developing cirrhosis and liver cancer. There is no effective treatment available for liver cirrhosis and cancer, so the development of alternative therapeutic strategies using a small animal model system to cure chronic HCV infection is crucial.

Development of novel antiviral therapies that target the multiple steps in the HCV life cycle, including viral genome replication, assembly, and infection is now possible due to the availability of the infectious, full-length HCV cell culture systems. A number of antiviral strategies have been developed during the past 2 years, including small molecules, anti-sense oligonucleotides, siRNA, ribozymes, and recombinant antibodies that have been designed to inhibit HCV replication in cell culture models[4,5]. Progress in the targeted delivery of these antiviral molecules to inhibit viral replication in the liver is hampered due to the lack of a small animal model for HCV infection. The development of an animal model for HCV infection has been difficult due to the fact that the virus has limited host tropism and can infect only humans and chimpanzees[6,7]. The chimpanzee is the only natural animal model for studying HCV infection[8]. The chimpanzee model has been used in the past to study many aspects of HCV pathobiology and infectivity using cloned viral genomes, and it remains an important animal model. However, it is a difficult and expensive animal model that cannot be used to optimize experiments that deal with the liver-targeted delivery of intracellular treatment approaches such as siRNA or antibodies. Therefore, a small animal model for HCV infection is required to test different experimental therapies developed using HCV cell culture systems. Rodents are the preferred animal models for testing gene delivery experiments because of their size, low cost and short gestation period. Previously, researchers have developed mouse models for HCV infection using transgenic technology or direct transfection of HCV RNA into the liver[9]. Transgenic mice are not models for HCV infection or antiviral testing because of stable integration of HCV cDNA into the mouse chromosome. It will be difficult to differentiate the viral RNA replication from the HCV RNA produced due to cellular transcription in the transgenic animal models. There has been limited success using this transgenic approach because mouse hepatocytes do not support HCV RNA replication or infection[9,10].

To overcome this limitation, four different mouse models have been developed to study experimental HCV infection *in vivo*: the immunotolerized rat model[11]; the Trimera mouse model[12]; the uPA/SCID mouse model[13-15]; and the Fah$^{-/-}$ Rag$^{-/-}$ IL-2$^{-/-}$ mouse model[16]. The rat model for HCV infection has been developed using immunotolerized rat embryos that can allow transplantation of human hepatoma cell lines that can be infected with HCV. These models have been used to study the complete life cycle of HCV infection, and antiviral testing. In the latter three models, researchers have used highly specialized mouse strains that support transplantation of human hepatocytes into the mouse liver. These models appear to be highly relevant animal models for HCV infection. A number of investigators have been using these models to infect chimeric mice with HCV or hepatitis B virus[13-16] However, these models are relatively complicated to use because they require specialized surgical skills, special strains of mice, use of human hepatocytes, as well as HCV-infected serum samples. Recently, a xenograft mouse tumor model for HCV infection has been described that utilizes the mouse-adapted replicon cell line that contains the luciferase reporter[17,18]. The replication of HCV RNA has been measured in the mouse liver and subcutaneous tumor xenograft using a whole-body real-time imaging system. This model has been used to evaluate the antiviral properties of IFN-α and protease inhibitors. There is a lot of interest in this mouse model because it is less expensive, non-infectious, and can be easily adapted to any research laboratory.

We describe here a mouse model for studying the replication of the JFH1 sub-genomic clone of HCV in subcutaneous and liver tumors using a green fluorescence protein (GFP)-labeled sub-genomic replicon in the Huh-7 cell line. The development of a mouse-adapted GFP-labeled replicon cell line, combined with the highly efficient JFH1 virus facilitates high-level replication of the HCV in subcutaneous and liver tumors of laboratory mice with a partially suppressed immune system. We have developed a mouse-adapted HCC cell clone that replicates sub-genomic HCV RNA and forms HCC xenografts in SCID mice. We show that replication of HCV in the subcutaneous and liver tumors can be assayed using several biochemical

methods by looking at GFP expression and evaluating the antiviral effects on HCV by cell colony assay, ribonuclease protection assay, and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). We showed that IFN-α successfully inhibited replication of HCV RNA in the HCC xenograft, which indicates that the model can be used to test the efficacy of different antiviral strategies that target the HCV replication cycle.

MATERIALS AND METHODS

Animals

Female SCID/bg and NOD/SCID γ mice 6-8 wk old were obtained from Charles River Laboratories (Wilmington, MA, USA) and Jackson laboratory (Bar Harbor, ME, USA), respectively. The mice were maintained in sterile conditions in a pathogen-free environment at the Department of Comparative Medicine, Tulane University Health Sciences Center. All animal experiments were carried out after receiving approval from the Institutional Animal Care and Use Committee (IACUC), Tulane University Health Sciences Center. All the SCID mice were γ-irradiated at 3 Gy (approximately 3.2 min) 1 d prior to cell transplantation. SCID/bg mice were used for subcutaneous tumor xenografts and NOD/SCID mice were used for liver tumor development. Throughout every experiment, we carefully checked the mice for their well-being, body condition and movement. All of the mice were weighed every alternate day to check for weight loss. A drop in their body weight was considered as an indication of sickness. After surgery, the mice were kept in separate sterile cages and observed carefully until they fully recovered.

Mouse-adapted replicon cell lines

We developed a stable Huh-7 cell line (S3-GFP) that replicated the HCV-GFP sub-genomic RNA of the JFH1 clone. The replicon cell line expressed a high level of GFP that could be visualized directly under a fluorescence microscope. We previously have shown that IFN-α inhibits replication of HCV in the S3-GFP cell line in a dose-dependent manner[19]. One million GFP-replicon cells were implanted subcutaneously into the right and left flank of SCID/bg mice. Mice were followed for the development of tumors. When the tumors reached 10 mm in size, they were harvested in a sterile Petri dish. Cells were dissociated by collagen digestion and separated by low-speed (500 rev/min) centrifugation. The cell pellet was resuspended in RBC lysis buffer (eBioscience, San Diego, CA, USA) for 15 min; cells were centrifuged and the cell pellet was resuspended in DMEM with 10% FBS supplemented with G-418 (1 mg/mL). The cells were cultured and expression of GFP in the Huh-7 cells was examined over time under a fluorescence microscope. When most of the Huh-7 cells in the culture showed GFP expression, they were harvested and injected into SCID mice for tumor development. The *in vivo* passaging experiments were repeated several times until > 50% of the cells in the subcutaneous tumor were GFP-positive.

Intrasplenic infusion of mouse-adapted replicon cells

Intrasplenic infusion of replicon cells was performed using a previously described procedure[20]. NOD/SCID γ mice were anesthetized with isoflurane under a laminar flow cabin. The surgical area was shaved and swabbed with betadine scrub. A small incision was made in the left flank in order to expose the spleen and carry out the cell injection. The spleen was accessed with a small forceps, and 10^6 replicon cells were injected into the inferior splenic pole; a monofilament suture was placed across the spleen at the site of injection to reduce spillage of cells into the abdominal cavity. The peritoneal wall and skin were separately closed using a monofilament suture and staples, respectively. After 3, 4, 5 and 6 wk, the animals were euthanized by CO_2 inhalation and their livers were removed. Part of the liver was fixed in 10% buffered saline for 72 h, processed, and embedded in paraffin. Tissue blocks were made for histological analysis after hematoxylin and eosin staining. The remaining part of the liver tissue was frozen in OCT compound for GFP expression analysis.

IFN treatment

IFN-α 2b (Intron A; Schering-Plough, NJ, USA) was diluted in PBS at a concentration of 150 IU/μL and stored at -70℃. Both the subcutaneous and liver tumor models were validated by intraperitoneal injection of 100 μL IFN-α solution (total 15 000 IU/mouse) three times weekly. A group of five mice was used to test the IFN antiviral effect in the subcutaneous and liver tumor models.

Histology and immunocytochemistry

The growth of HCC xenografts in the SCID mice was examined by hematoxylin and eosin staining of fixed and paraffin-embedded mouse tumor and liver specimens. Five-micrometer sections were cut from each tissue block, mounted on a glass slide, and dried over night at room temperature. All of the sections were deparaffinized in xylene, rehydrated by dipping in a graded alcohol series, and washed in PBS. To demonstrate the implantation of replicon cells in the liver, the tissue sections were stained with an antibody against human serum albumin (Dako, Carpinteria, CA, USA). The immunoreactivity of the albumin antibody was detected using the ABC detection kit using a standard laboratory protocol. To demonstrate expression of GFP in the subcutaneous and liver tumors, frozen sections were prepared. The sections were washed in PBS and stained with Hoechst dye (H33342, Calbiochem, Germany). Expression of GFP in the HCC xenograft was observed using a fluorescence microscope (Olympus) using a standard procedure[19].

Cell colony assay

To study the replication of HCV sub-genomic RNA in the HCC xenografts, the liver was digested with collagenase and viable S3-GFP replicon cells were obtained by low-speed centrifugation. The cell pellet was suspended in 5 mL RBC lysis buffer (eBiosciences) for 15 min. The

viable tumor cells (S3-GFP) were cultured in DMEM supplemented with G-418 (1 mg/mL). Huh-7 cells that supported HCV RNA replication after IFN treatment were selected. The number of cell colonies formed in each cell culture dish was counted after Giemsa staining (Sigma, St. Louis, MO, USA).

RPA

Total RNA was isolated from the subcutaneous tumor by the GITC method and subjected to RPA for the detection of genomic positive-strand HCV RNA, using an antisense RNA probe that targeted the 5' untranslated region (UTR). For the RPA, 25 μg total RNA was mixed with a negative-strand RNA probe that targeted the 5'-UTR of HCV (10^6 cpm) in a 10-μL hybridization solution, denatured for 3 min at 95℃, and hybridized overnight at 50℃. RNase digestion was performed in 200 μL RNase digestion buffer (10 mmol/L Tris, pH 7.5, 5 mmol/L EDTA and 0.3 mol/L NaCl) that contained RNaseA/T1 cocktail at 1:100 dilutions (Ambion, Austin, TX, USA) for about 1 h at 37℃. It was then treated with 2.5 μL 25% SDS and 10 μL proteinase K (20 mg/mL) for 15 min. Samples were extracted with phenol:chloroform and precipitated with absolute ethanol. The pellet was suspended in 16 μL gel loading buffer, heat denatured, and separated on a 6% TBE-urea gel (Invitrogen, Carlsbad, CA, USA). The gel was dried and exposed to X-ray film (Kodak Biomax-XAR, Rochester, NY, USA). To detect JFH1-HCV mRNA in the transfected cells, we prepared a plasmid construct called pCR-II-2a (Invitrogen), which contained the sequence of 79-297 nt of the 5'-UTR sequence of the JFH1 clone (pCR-II NT-218). This plasmid was linearized with the *Xba*I restriction enzyme. T7 RNA polymerase was used to prepare a negative-strand RNA probe for detection of positive-strand HCV RNA. The same amounts of the RNA extracts were subjected to RPA for GAPDH mRNA. We used a linearized pTRI-GAPDH-human antisense control template to prepare a probe to detect GAPDH mRNA using Sp6 RNA polymerase (Ambion). The appearance of a 218-nt fragment in the RPA indicated the presence of HCV positive-strand RNA.

RT-nested PCR and Southern blot analysis

To detect HCV RNA in the liver tumors, total RNA was isolated from the liver tissues by the GITC method[21]. cDNA synthesis for HCV positive-strand RNA was carried out using 1 μg total cellular RNA, an outer antisense primer and avian myoblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). Amplification of the cDNA was performed in 50 μL reaction mixture that contained 250 ng outer sense primer and *Taq* DNA polymerase. The first PCR product was amplified by another round of PCR using outer and inner sets of primers, as described previously[21]. The HCV PCR products were further subjected to Southern blot analysis by using a positive-sense HCV-specific ^{32}P-labeled oligoprobe. One microgram of total cellular RNA was used to amplify albumin mRNA levels by RT-PCR. The sequence of primer and probe used in these_experiments has been described previously[21].

Real-time RT-qPCR

Real time RT-qPCR was performed to quantify HCV RNA levels in the infected cell culture using a published protocol[22]. The 243-bp HCV DNA was amplified from the RNA extract by RT-PCR using the outer sense primer 5'-GCAGAAAGCGCCTAGCCATGGCGT-3' (67-90) and outer antisense primer 5'-CTCGCAAGCGCCCTATCAGGCAGT-3' (287-310). cDNA synthesis was performed from positive-strand HCV RNA using an outer antisense primer that targeted the highly conserved 5'-UTR region of HCV in a 20-μL volume. Two micrograms of total cellular RNA were mixed with 1 μL outer antisense primer (200 ng/μL), denaturized at 65℃ for 10 min, and annealed at room temperature. AMV reverse transcriptase (10 U) (Promega) was added and incubated at 42℃ for 60 min in the presence of 50 mmol/L Tris, pH 8.3, 50 mmol/L EDTA, 500 nmol/L dNTP, 250 nmol/L spermidine, and 40 U RNasin (Promega). cDNA was stored at -20℃ until use. SYBR Green real-time PCR amplification was performed in a 20-μL volume that contained 10 μL SYBR Green ER qPCR SuperMix, 1 μL (250 ng/μL) of sense and antisense primer with 4 μL cDNA and 4 μL distilled water. All samples were run in triplicate. The amplification was carried out using the standard program recommended by Bio-Rad Laboratories that included: a first cycle at 50℃ for 2 min, 95℃ for 8 min, and an additional 50 cycles in which each cycle consisted of a denaturation step at 95℃ for 10 s, and annealing and extension steps at 60℃ for 30 s. At the end of the amplification cycles, melting temperature analysis was performed by a slow increase in temperature (0.1℃/s) up to 95℃. Amplification, data acquisition, and analysis were performed on CFX96 Real Time instrument (Bio-Rad Laboratories) using CFX manager software (Bio-Rad Laboratories).

RESULTS

HCC xenografts formed subcutaneously support HCV replication

We prepared a chimeric clone of the sub-genomic HCV of JFH1 2a virus by fusing it with GFP in a frame with one of the non-structural proteins of HCV (NS5A). Huh-7 replicon cells that replicated the HCV-GFP subgenomic RNA emitted a green fluorescent signal when exposed to a specific wavelength of light. This allowed direct visualization of HCV RNA replication. These replicon cells were cultured for > 1 year and were found to have stably maintained GFP expression with > 60%-80% of cells positive by flow analysis. We have shown that IFN-α inhibits replication of HCV RNA and inhibits GFP expression in a dose-dependent manner in the replicon cells[19]. With this GFP-based replicon cell line, we sought to establish an HCC xenograft in SCID mice using the mouse-adapted replicon cells as described previously[18]. The experimental steps involved in generating the *in vivo*

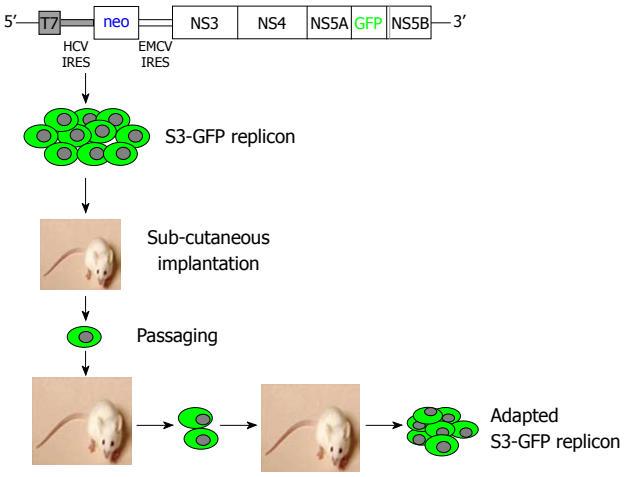

Figure 1 Summary of overall experimental plan to generate a mouse-adapted hepatitis C virus-green fluorescence protein replicon cell line. A chimeric replicon clone was prepared by inserting the green fluorescence protein (GFP) coding sequences with the NS5A sequences of the hepatitis C virus (HCV) sub-genomic clone. Huh-7 cells were transfected with a transcribed sub-genomic HCV-RNA replicon. A stable Huh-7 cell line with replicating HCV GFP chimera RNA (S3-GFP replicon) was developed. The replicon cell line was implanted in SCID mice for tumor development. Subcutaneous tumor that developed in SCID mice was collected and cells with replicating HCV-GFP were selected by culture in growth medium that contained G-418 (1 mg/mL). The *in vivo* adaptation process was repeated to generate a mouse-adapted S3-GFP replicon cell line that demonstrated 50% GFP expression in subcutaneous tumors.

adapted GFP replicon cells are summarized in Figure 1. S3-GFP replicon cells were injected subcutaneously into the right and left flank of SCID mice after γ irradiation. Tumors were harvested when they were 10 mm in size and cells were isolated and cultured in selection medium. After the first passage, we found that expression of GFP in the replicon cells was very faint and only a few cells (< 10%) showed bright GFP positivity. The sub-genomic HCV RNA has a neomycin gene selection marker that allows the selection of tumor cells that support HCV replication. Therefore, replicon cells were cultured with medium that contained G-418, to select for increased intracellular GFP expression. After 1 wk, a high level of GFP expression was seen in most of the cells in culture. This experiment suggested that *in vivo* tolerance occurred at the cellular level as well as at the level of intracellular virus replication in the replicon cells. We were able to select a homogeneous population of Huh-7 cells with stable GFP expression after the first passage in SCID mice. To obtain a GFP replicon cell line with increased growth and replication capacity, *in vivo* passaging experiments were repeated three additional times. Figure 2 suggests that *in vivo* passaging of replicon cells allowed for the selection of replicon cells with increased replicative ability. The expression of GFP in the frozen tumor sections was examined under a fluorescence microscope after nuclear staining. The levels of viral RNA replication in the subcutaneously formed HCC xenografts were very high and GFP expression was observed in most of the replicon cells (Figure 2). We generated a highly adapted GFP replicon cell line (S-3GFP/*in vivo*) that formed a HCC xenograft in SCID/bg mice within 2 wk.

HCC xenografts in the liver support HCV replication

It is important to develop an HCC xenograft in the liver because HCV replicates in the liver of the human host. Furthermore, a liver tumor model is needed to assess and optimize the targeted delivery of antiviral therapy. We wanted to determine whether the mouse-adapted replicon cells that formed a subcutaneous tumor could also form HCC in NOD/SCID mouse liver. The Huh-7 cells were infused into the liver by intrasplenic injection, and examined for liver tumor development at weekly intervals. The growth of mouse-adapted Huh-7 cells in the liver was slow compared to the subcutaneous model, and took almost 4 wk to reveal diffuse HCC nodules throughout the liver (Figure 3C and D). Normal mouse liver and histology are shown in Figure 3A and B. To examine whether HCV replication in liver tumors occurred at a similar level as that seen in the subcutaneous model, frozen sections of the liver tumors were examined for GFP expression. To our surprise, the level of HCV RNA replication was low in the liver tumor, and we could not see a very high level of GFP expression in the HCC xenografts formed in the SCID mouse liver. The loss of GFP expression was not due to an alteration in the open reading frame of GFP in the sub-genomic HCV RNA, because we could recover HCV GFP RNA levels when cells were isolated from the xenografts and cultured in medium supplemented with G-418. The negative GFP expression was due to the low level of HCV replication in the liver tumors. These results suggested that the liver microenvironment significantly suppressed HCV RNA replication compared to that in the subcutaneously formed tumors. We then examined whether these HCV GFP replicon cells needed to adapt to the liver microenvironment to support high-level replication of HCV. The mouse-adapted GFP replicon cells were therefore adapted to the liver microenvironment by another three passages. At each passage, we could recover HCV GFP expression in culture, which suggested that HCV RNA replication in the liver tumors was not completely lost. We then examined whether GFP expression could be seen in the HCC xenografts formed in the mouse liver, using the cells that had undergone three passages. We were able to see GFP expression in tumor nodules in the liver after three passages (Figure 3E and F). The replication of HCV RNA in the liver tumors was also confirmed by cell colony assay after culturing tumor cells in medium that contained G-418 (1 mg/mL). The replicon cells derived from the liver tumors formed G-418-resistant cell clones, which suggested that HCV replication occurred within the HCC xenografts in SCID mice. Liver tumor supporting HCV RNA replication was confirmed by RT-nested PCR followed by Southern blot analysis. These results suggest that we have developed a mouse-adapted S3-GFP replicon cell line that forms liver tumor after splenic injection. The level of HCV RNA in the liver tumor was determined by real time RT-qPCR that was in the range of 10^4 copies/μg of total cellular RNA. The subcutaneous tumor tissue showed a mean titer of 10^5 copies/μg of total cellular RNA. There is one log reduction in the HCV RNA titer in

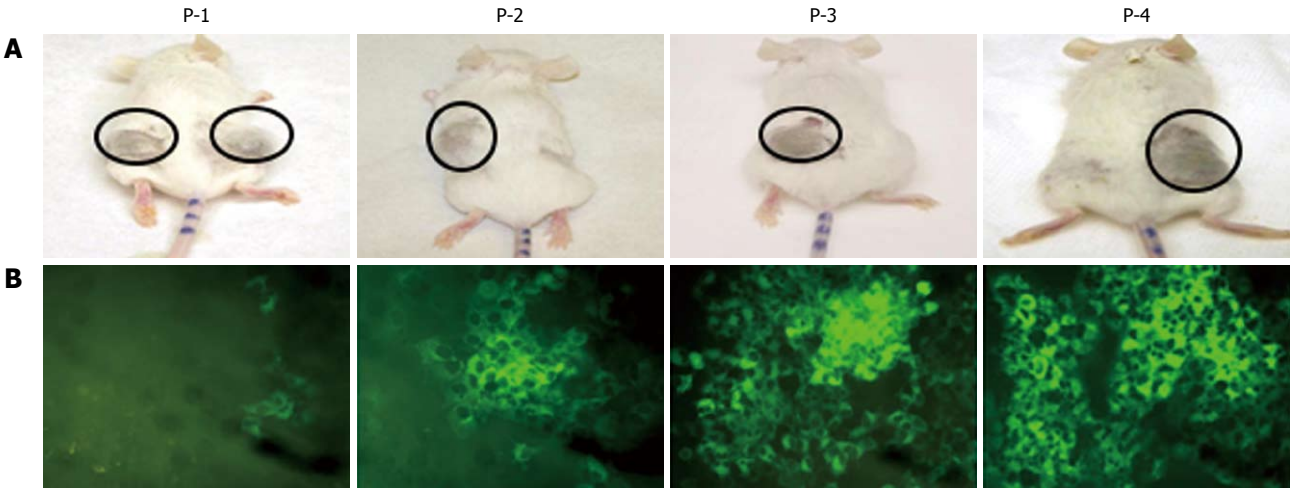

Figure 2 Intracellular expression of hepatitis C virus-green fluorescence protein in the subcutaneous tumor of SCID mice. Mice were injected subcutaneously with 10^6 hepatitis C virus-green fluorescence protein (GFP) replicon cells. Tumor growth was monitored on a weekly basis. A: Tumorigenicity of Huh-7 replicon cells in γ-irradiated SCID mice; B: Expression of GFP in tumor cells during the *in vivo* passage. The number of GFP positive cells was low (< 10%) after the first passage. There was a gradual increase in GFP expression in the subcutaneous tumors after each passage. After four passages, the number of GFP-positive cells increased significantly (> 50%).

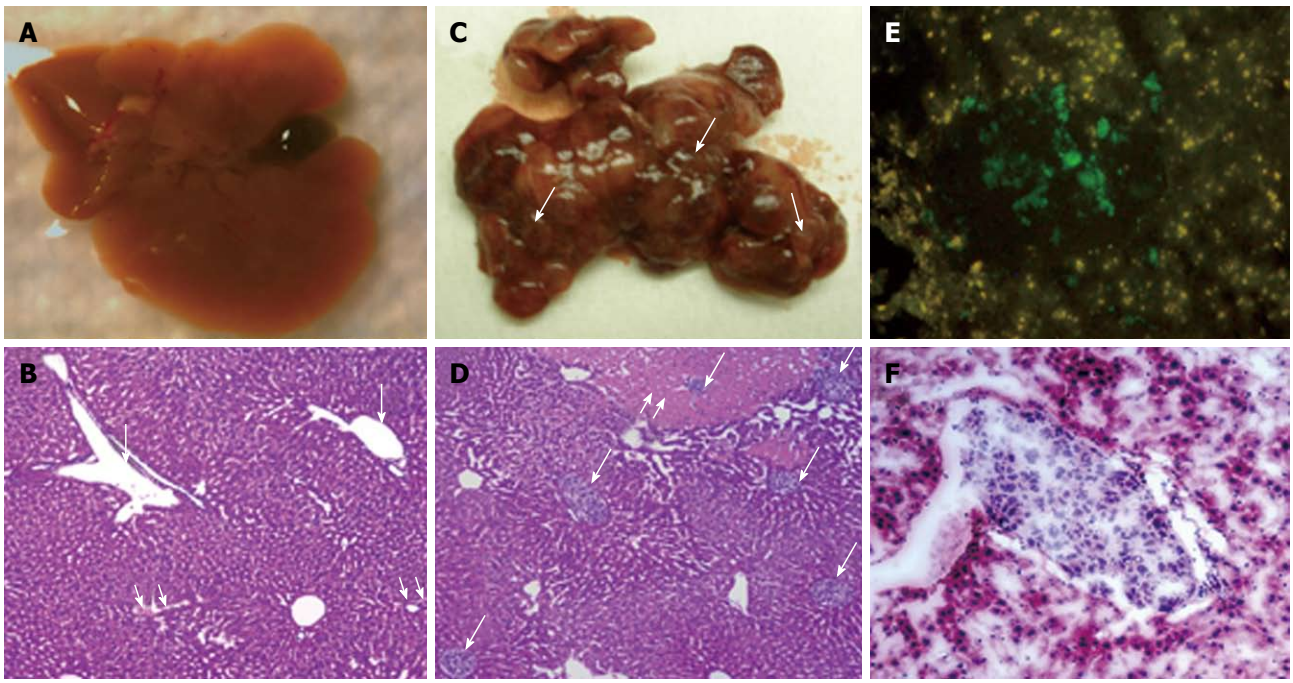

Figure 3 Human hepatocellular carcinoma xenograft in SCID/NOD mouse liver. A: Gross appearance of normal mouse liver with distended gall bladder; B: Light microscopic appearance of normal liver stained with hematoxylin and eosin (4 × magnification). The portal tracts are shown by single arrows and the central veins are marked with double arrows; C: Gross appearance of mouse liver with metastatic nodules of hepatocellular carcinoma (HCC). Note the distended white-tan areas of metastasis and infarction in the mouse liver (single arrows); D: Microscopic metastasis of HCC diffusely infiltrating the liver through the portal venous system (single arrows). Note the areas of infarction secondary to the tumor emboli in the portal vein (double arrow). Human hepatocytes can be easily discriminated from the mouse hepatocytes by their size and pale color; E: Expression of hepatitis C virus-green fluorescence protein (GFP) fusion protein in the S3-GFP liver tumors in the mouse; F: Hematoxylin and eosin staining (frozen section) of HCC tumor in the mouse liver at 4 wk after intrasplenic infusion of S3-GFP replicon cells.

the HCC tumor formed subcutaneously *vs* those formed in the liver (Figure 4). The HCV RNA titer in the liver tumor model is comparable to that found in chronically infected human liver tissue[23]. We hypothesize that this might be due to the fact that there are differences in the innate immune pressure on HCV RNA replication in the peripheral compared with internal organs such as the liver. There is evidence to support our observations that the liver has its own innate immune system that might suppress replication of HCV[24-26].

IFN-α inhibits HCV replication in the HCC xenograft formed subcutaneously

To investigate the ability of IFN-α to inhibit HCV RNA

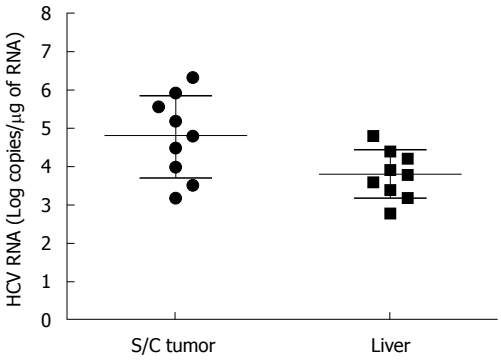

Figure 4 Comparison of hepatitis C virus RNA level between hepatocellular carcinoma xenografts formed subcutaneously and in the liver. RNA extract was prepared using the GITC method. The hepatitis C virus (HCV) RNA level was measured by real-time polymerase chain reaction using 1 μg total RNA isolated from the tumor samples, and mentioned as log copies/μg RNA. HCV RNA levels were 10-fold higher in the tumors formed subcutaneously compared to those that were formed in the liver. The titer of HCV in the liver tumor model was comparable to that in the infected human liver.

replication in the subcutaneous HCC xenografts, the mice at 2 wk post-implantation were treated with IFN-α at 15000 IU/mice three times weekly. The mice were sacrificed at different time points after initiation of IFN-α treatment and the tissues were processed for HCV expression. IFN treatment decreased GFP expression in a time-dependent manner, with > 50% decrease in GFP expression after 1 wk (Figure 5B and E), with complete inhibition after 2 wk (Figure 5C and F). The untreated sections showed expression of GFP (Figure 5A and D). To show that the hepatocytes that support HCV replication are human hepatoma cells, immunostaining of HCC cells was performed using an antibody to human serum albumin. The results presented in Figure 5G-I clearly showed that the HCC xenografts were positive for human albumin. The level of albumin expression did not change due to IFN-α treatment. To ensure that IFN-α treatment inhibited viral RNA replication and reduced the level of HCV RNA in the HCC xenografts, total RNA was extracted from the tumor samples and processed for RPA. The results of this analysis are shown in Figure 6, which indicated that HCC xenografts in SCID mice supported a high level of HCV RNA replication, and the levels of HCV RNA in the tumors were completely inhibited after 2 wk of IFN-α treatment. The results shown in Figure 6A and B suggested that HCV RNA level in the subcutaneous HCC tumors increased from 18 to 28 d after tumor development. IFN treatment successfully inhibited virus replication within the tumors and the HCV-RNA titer decreased significantly after 2 wk of treatment, and remained below the limits of detection at 4 wk. We examined the possibility that IFN-α treatment could have an effect on tumor growth, which could have affected the measured HCV RNA levels in the tumors. The tumor size and histology of IFN-α-treated and untreated animals were compared (Figure 7). We did not notice any difference in the tumor growth or histology between the treated and untreated animals. The dose of IFN-α treatment used in the *in vivo* experiments only inhibited HCV RNA replication, but did not inhibit the tumor growth. Taken together, these results indicated that HCC xenografts that formed subcutaneously in the SCID mice supported a high level of HCV RNA replication, and that IFN-α directly inhibited HCV replication in the tumors. All mice were doing well throughout the IFN-α treatment experiment and their body weight did not change significantly. Mice tolerated the IFN-α treatment well.

IFN-α completely inhibits replication of HCV 2a subgenomic RNA in the HCC xenograft in mouse liver

We demonstrated the usefulness of this model by evaluating whether HCV replication in the liver tumor model could be inhibited by IFN-α. Mice were treated with intraperitoneal injection of IFN-α three times weekly at 15000 IU/mouse, starting at 4 wk after S3-GFP transplantation. Control animals received saline injections at similar time intervals. At different treatment intervals, mice were sacrificed and liver tissue was collected. The ability of IFN-α to inhibit HCV replication was confirmed by four independent assays. First, hepatocytes were isolated from the liver and cultured in the presence of growth medium that contained G-418 (1 mg/mL). The number of S3-GFP replicon cell colonies in the plate was compared to assess the antiviral effects of IFN-α against HCV replication in the xenograft tumors. Figure 8A shows that 4 wk IFN-α treatment successfully inhibited replication of HCV in the liver tumors. Second, the intracellular HCV RNA in the tumors tissue was examined by using RT-nested PCR and Southern blot analysis that targeted the highly conserved 5'-UTR region of the HCV genome. We showed that HCV RNA was detectable in the liver tumors of untreated mice over 5 wk, by RT-nested PCR and Southern blot analysis. IFN-α treatment completely inhibited HCV RNA replication in the liver tumors and remained undetectable at 4 and 5 wk, using the highly sensitive RT-nested PCR and Southern blot analysis (Figure 8B). IFN-α treatment did not alter the cellular albumin RNA levels. Third, expression of GFP in the liver tumors after IFN-α treatment was negative after 4 wk (Figure 8C). Fourth, the level of HCV RNA in the treated and untreated tumor samples was assessed by real-time PCR. The results of this analysis suggested that HCV levels decreased significantly in the tumors and remained below the detection limit after 4 wk of IFN-α treatment (Figure 8D). These data clearly showed that IFN-α inhibited HCV RNA replication in the liver tumor model and there was a significant difference in the level of HCV replication in the liver tumors between the untreated and IFN-α-treated mice. To demonstrate that the decrease in the HCV RNA replication after IFN-α treatment was not due to the direct effect of IFN on tumor growth in these HCC xenografts, histological examination of liver tumor in IFN-α-treated and untreated animals was performed. The histological pictures shown in Figure 9 indicate that there was no evidence of cellular necrosis in the tissue

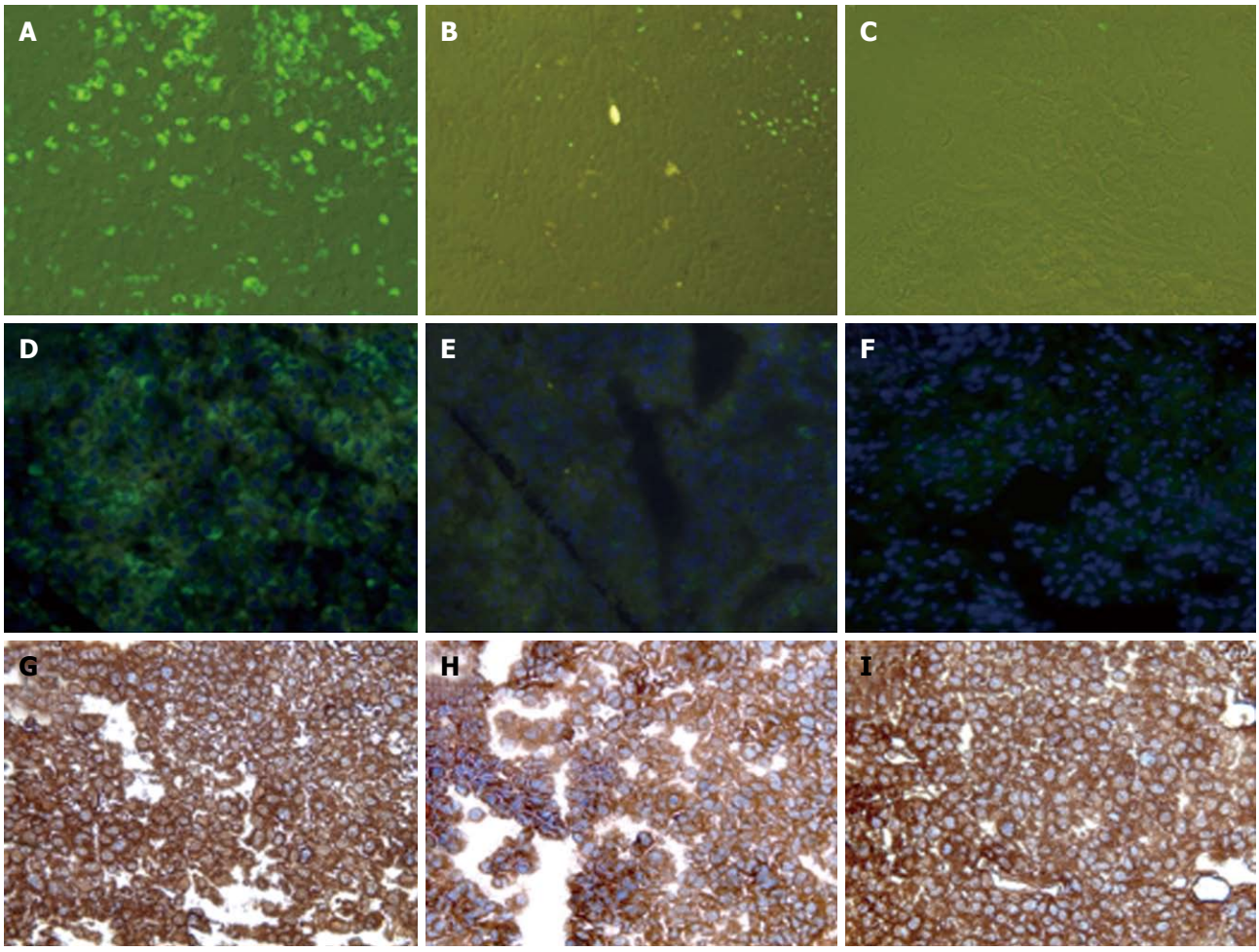

Figure 5 Interferon-α inhibits hepatitis C virus-green fluorescence protein expression in human hepatocellular xenografts formed subcutaneously in SCID mice. Mouse-adapted replicon cells were injected subcutaneously into SCID mice, which then developed visible tumors after 2 wk. The mice were injected intraperitoneally with a total dose of 15 000 IU interferon-α (IFN-α) in 100-μL volumes, three times weekly. Tumors were harvested after 1 and 2 wk of IFN treatment and examined for green fluorescence protein (GFP) expression and viral RNA by RPA and real-time polymerase chain reaction. A, D: Expression of GFP in the frozen sections of hepatocellular carcinoma (HCC) xenografts before IFN-α treatment; B, E: Expression of GFP 1 wk after IFN-treatment; C, F: Expression of GFP after 2 wk IFN-treatment. The middle panel shows DAPI staining of the nucleus. IFN inhibited hepatitis C virus RNA replication and GFP expression in the liver tumors at 7 and 14 d; G-I: Intracytoplasmic expression of human albumin in the HCC xenografts formed by subcutaneous injection of *in vivo* adapted Huh-7 replicon cells.

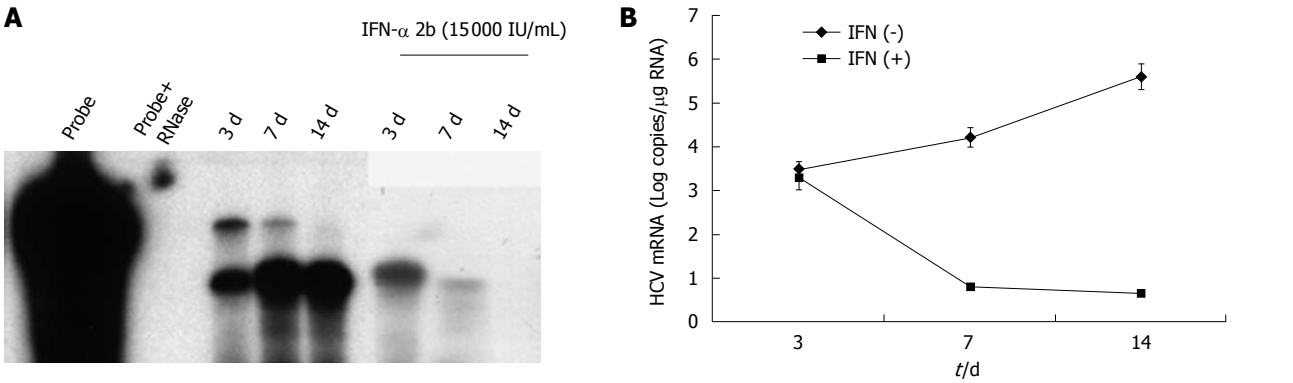

Figure 6 Intracellular hepatitis C virus RNA levels in subcutaneous tumors. A: RPA measuring intracellular hepatitis C virus (HCV) RNA levels in hepatocellular xenografts formed subcutaneously. There was a gradual increase in HCV RNA levels in the subcutaneous tumors at 3, 7 and 14 d post-tumor development. Two weeks of interferon (IFN) treatment completely inhibited HCV RNA replication in the subcutaneous tumors, as demonstrated by a negative RPA signal at 14 d of IFN-α treatment; B: Real-time reverse transcription polymerase chain reaction showing HCV RNA levels in the subcutaneous tumors before and after IFN-α treatment. IFN treatment decreased HCV RNA levels in the tumors, which remained below the detection limits after 2 wk.

sections of mice that received IFN-α treatment. These results indicated that the reduction of HCV RNA levels in the tumor was due to a specific antiviral mechanism of IFN-α.

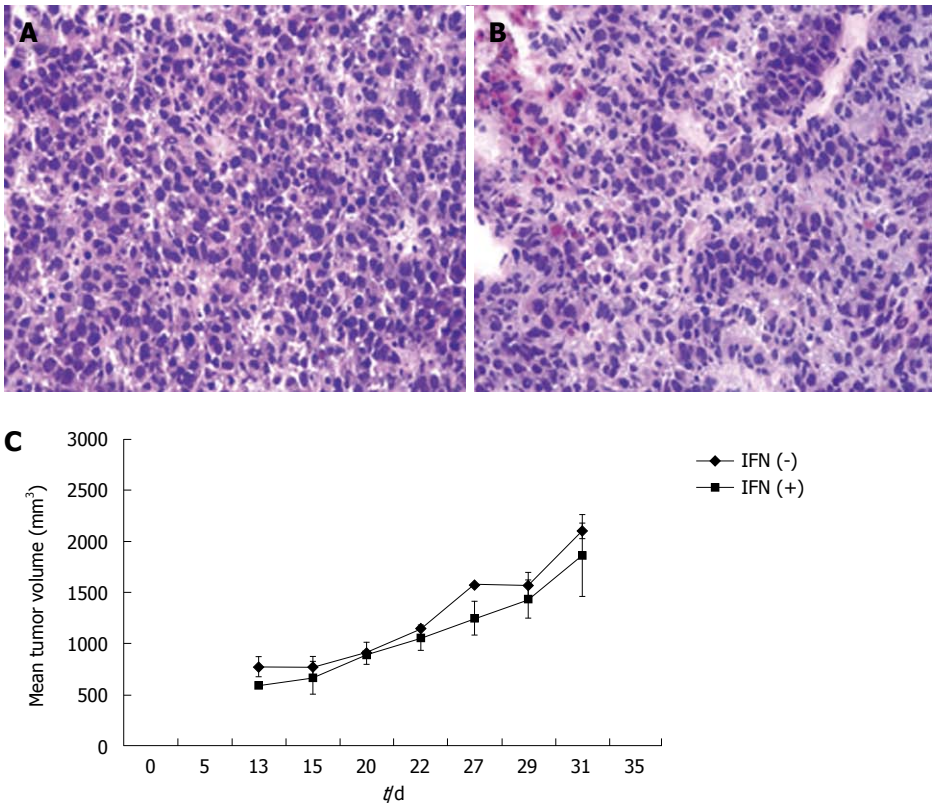

Figure 7 Interferon treatment has no effect of tumor growth in SCID/bg mice. A, B: Histology of tumor sections of experimental mice before (A) and after (B) 2 wk of interferon-α (IFN-α) treatment showed no evidence of tumor cell necrosis or apoptosis (10 × magnification); C: Kinetics of tumor growth between two groups of mice during IFN-α treatment. The mean tumor volume remained the same between these two groups of mice.

DISCUSSION

Cell culture and animal models are required for the development of novel experimental therapies for chronic HCV infection. A number of antiviral strategies have been developed that inhibit HCV replication in cell culture systems[4,5]. The successful use of different therapeutic agents to inhibit chronic HCV infection requires further validation using an easily accessible small animal model. Our laboratory has developed multiple siRNA targets in the 5'-UTR region of the HCV genome, and has shown that intracellular delivery of these siRNAs can completely degrade HCV RNA in cell culture[27]. We have also developed recombinant antibodies targeted to the viral NS3 helicase, and have shown that intracellular expression of these antibodies inhibits viral helicase activity and virus replication in a cell culture model[28]. The next step of this research is to test the effectiveness of these different antiviral strategies that target inhibition of viral replication, using an animal model system. Therefore, development of a small animal model for HCV infection was essential to optimize targeted, hepatic delivery of siRNA and recombinant antibodies. Mice and other rodents are not susceptible to natural HCV infection, thus, there are several alternative approaches that have been employed during the past few years to replicate HCV infection using mouse models. The advantages and disadvantages of these models to study HCV infection in mouse models are discussed.

The most promising small animal model for studying HCV infection is now the human liver chimeric mouse. The principle of developing this mouse model is based on the availability of specific mouse strains in which human hepatocytes can be efficiently transplanted into the mouse liver. Two specific mouse strains are currently being used for development of the humanized mouse liver. One is the SCID-Alb/uPA mouse and the other is the Fah$^{-/-}$ Rag$^{-/-}$ IL-2$^{-/-}$ mouse. Both mouse strains have high liver regeneration capacity due to their liver injury and support a high degree of human hepatocyte transplantation. The humanized mouse liver has been shown to have normal hepatic architecture and support HCV replication after natural infection. The studies conducted by Mercer *et al*[13] and Tateno *et al*[29] have used a mouse strain called SCID-Alb/uPA. Using this mouse strain, Mercer *et al*[13] have shown that the humanized mouse liver contains > 50% human hepatocytes, and Tateno *et al*[29] have shown that humanized mouse liver contains > 92% human hepatocytes. The Alb/uPA transgenic mouse strain shows severe hepatotoxicity due to overexpression of the urokinase plasminogen (*uPA*) gene in hepatocytes, which leads to continuous liver regeneration. Human hepatocytes that lack this transgene preferentially survive after transplantation and are stably maintained in this mouse liver. This mouse strain has been crossed with an immunodeficient mouse (SCID or Rag2) to generate the SCID-Alb/uPA mouse strain that tolerates transplantation of human hepa-

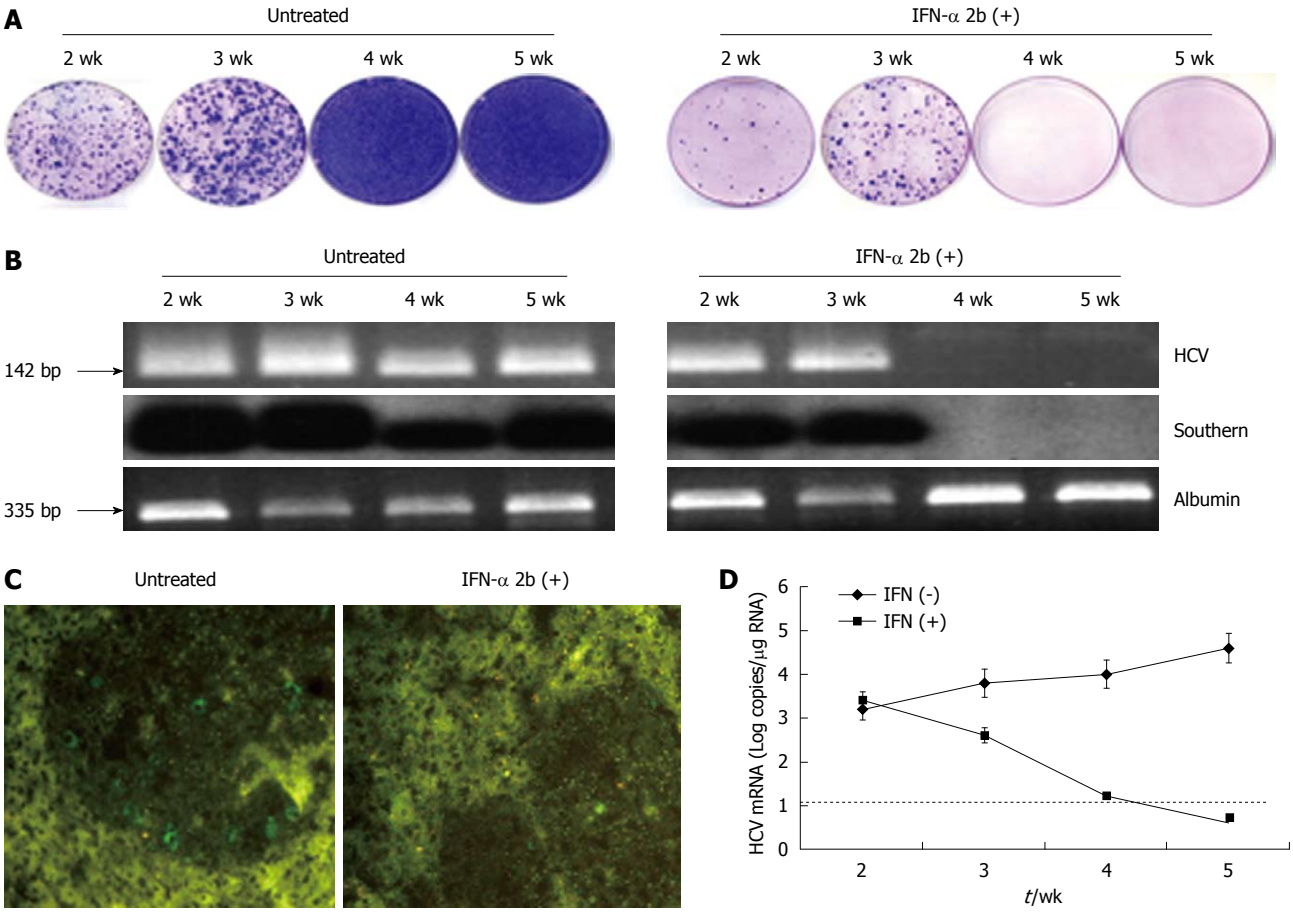

Figure 8 Antiviral effect of interferon-α in the liver tumor model. S3-green fluorescence protein (GFP) cells were implanted in the liver of NOD/SCID mice by intrasplenic injection. After 3 wk, interferon-α (IFN-α) treatment was started. The time shown represents the weeks after IFN treatment. A: Colony assay of viable tumor cells isolated from the liver tumor model at different time points with or without IFN-α treatment. Tumor cells isolated from the entire mouse liver were cultured in the medium that contained 1 mg/mL G-418. The replicon cells with hepatitis C virus (HCV) survived the treatment and formed cell colonies. Left panel: there was an increase in the number of cell colonies between 4 and 5 wk. Right panel: IFN-α treatment completely inhibited HCV replication and cell colony formation at 4 wk; B: RT-nested polymerase chain reaction (PCR) and Southern blot analysis for HCV and albumin in the RNA extracts of the liver tumor. Left panel shows the results without treatment. Right panel shows IFN-α treatment; C: Expression of HCV-GFP in the liver tumors before and after IFN treatment. IFN treatment after 4 wk completely inhibited HCV-GFP expression in the S3-GFP tumors in the SCID mouse liver; D: Real-time reverse transcription PCR (RT-PCR) showed that the levels of HCV in the liver tumor remained undetected after 4 wk of IFN-α treatment. The dotted line indicates the limit of detection for real time RT-PCR assay.

tocytes well. Human hepatocytes can be successfully transplanted into the SCID-Alb/uPA mice within 1 or 2 wk after birth. Several studies have now successfully used this model to replicate HCV infection and have tested several antiviral molecules, including IFN-α and protease inhibitors. The study conducted by Bissig *et al*[16] has used Fah$^{-/-}$ Rag2$^{-/-}$ IL-2$^{-/-}$ mice and has shown that the humanized mouse liver contains > 80% human hepatocytes. This mouse strain lacks fumaryl acetoacetate, which is an enzyme that is required for tyrosine amino acid metabolism. Mice that lack this enzyme show severe hepatotoxicity due to accumulation of toxic metabolites. This mouse strain has been bred with Rag2$^{-/-}$ IL-2$^{-/-}$ mice to generate an immunodeficient mouse strain that supports transplantation of adult human hepatocytes. Recently, Bissig *et al*[16] have used this mouse model to replicate HCV infection from cell culture and from the serum of chronically infected patients. They also have shown that pegylated IFN-α and Debio 025 (cyclophilin inhibitor) inhibit HCV replication in this chimeric mouse model. The investigators have shown that these chimeric mice sustain viral replication for > 6 mo, without any substantial side effects. There is no doubt that these chimeric mouse models are extremely challenging technically, complicated to use, and expensive. Despite the technical challenges, these models hold promise for studying the long-term effect of HCV infection on the development of liver fibrosis and cancer.

We described here an HCC xenograft mouse model in which human tumor cells were implanted in the liver of an immunodeficient mouse. This model is relatively simple compared to the human liver chimeric mouse model. This model can be used for experimental verification of the efficacy of targeted antiviral therapies against HCV. The model utilizes SCID/bg and NOD/SCID mice with a partially suppressed innate immune system (natural killer cell response) and adaptive immune system (both B- and T-cell mediated response)[30]. In an earlier study, Zhu *et al*[18] have performed similar experiments to generate a mouse-adapted HCV Con-1 replicon cell line using the SCID/bg mice. The *in vivo* adaptation of HCV is not possible using

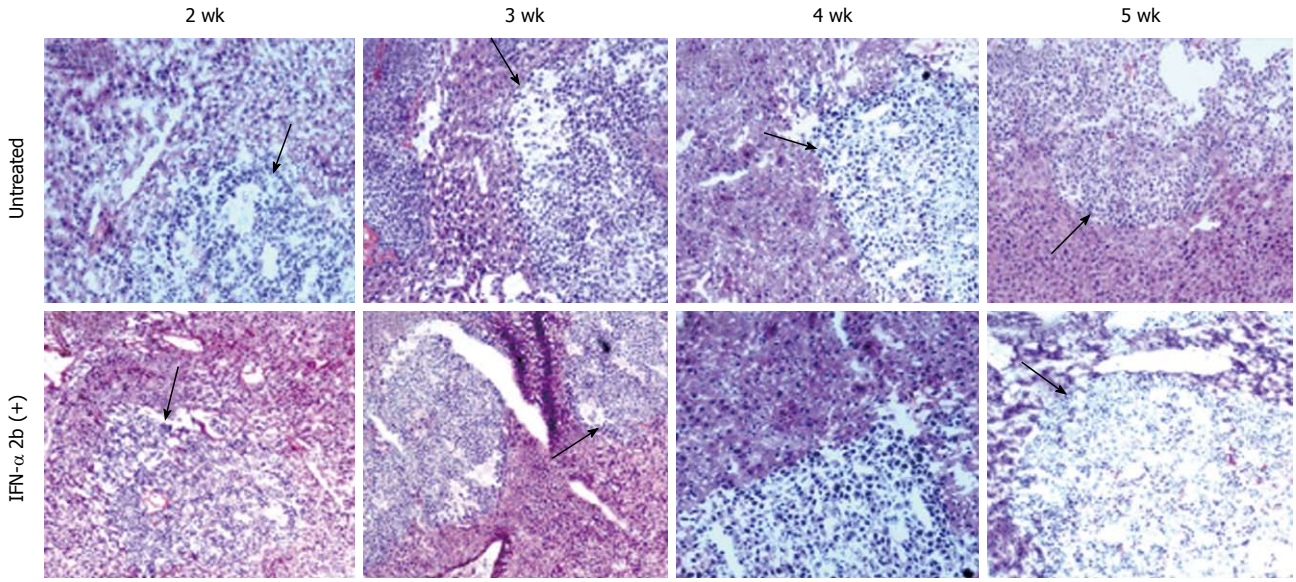

Figure 9 Histological evaluation of liver sections after hematoxylin and eosin staining that indicates that interferon-α treatment did not cause any tumor necrosis or reduce the size of tumor nodules in the SCID mice liver. The upper panel shows the untreated mouse liver sections after 2-5 wk of tumor development. The black arrows indicate the hepatocellular carcinoma (HCC) tumor in the mouse liver. Similarly, the lower panel shows the interferon-α-treated mouse liver sections with HCC tumor at different time points (10 × magnification).

this approach due to the low-level replication capacity of the HCV Con-1 replicon clone. The authors had to re-engineer the sub-genomic clone with reinsertion of HCV sub-genomic RNA with a highly efficient internal ribosome entry site, and a highly adapted mutation in the non-structural gene, to sustain high-level replication of HCV RNA within the tumor. They have successfully measured HCV replication in this mouse model by measuring luciferase activity using non-invasive whole-body imaging. They have shown that IFN-α and protease inhibitor BILN-2061 reduce HCV RNA replication in the subcutaneous and liver tumor models. The authors have not shown whether these treatments lead to complete clearance of HCV in these tumors models. Another study by Guévin *et al*[31] also has demonstrated that full-length HCV replication can be achieved in HCC tumor xenografts formed subcutaneously using immunodeficient mouse strains. The authors have made mouse-adapted Huh-7 cells by multiple passages in SCID mice. The authors have shown that the xenograft tumors in the SCID mouse produce infectious virus particles. The infectious virus produced in the xenograft tumors can be serially passaged in SCID mice. The authors have shown that IFN-α and protease inhibitor BILN-2061 inhibit replication of the cell culture HCV cc strain JFH1 in the mouse model.

The mouse model described here differs in some aspects compared to the two previously described tumor xenograft models. We realized that high-level replication of HCV in the SCID mouse liver might be possible by using the highly efficient JFH1 clone. We used the JFH1 virus clone that replicated at a much higher level in the HCC xenografts formed in the SCID mice. The GFP-labeled replicon cell line showed that replication of HCV occurred in the HCC tumors, and could be determined by direct examination under fluorescence microscopy. The replicon cell line is similar to chronically infected human hepatocytes, except that these cells do not produce the infectious virus, which makes this animal model safer to use. We have used a number of biochemical methods (colony assay, RPA, real-time RT-PCR and RT-nested PCR and Southern blot analysis) that quantitatively evaluate HCV replication in the subcutaneous tumors in an accurate manner. If necessary, this model can also be adapted using a luciferase-based, mouse-adapted HCV replicon cell line so that the whole-body imaging technique can be used to monitor HCV replication in the mouse model. The diffuse metastasis of HCC throughout the liver lobes after intrasplenic infusion of replicon cells mimics *in vivo* distribution of chronically infected hepatocytes in human liver. We demonstrated replication of HCV RNA in multiple HCC nodules in the portal tracts of the SCID mice livers. This liver tumor model developed by intrasplenic injection of replicon cells provides a better assessment of the biodistribution and pharmacokinetics of IFN-α when compared to direct injection of replicon cells to the liver, where HCC tumors are formed only in selected areas of the organ. We noticed that the level of HCV replication in the liver tumor model was 10-fold lower when compared to that in the subcutaneous model. This could be due to the innate antiviral pressure against HCV between the peripheral organ and liver microenvironment. The dynamics of viral RNA replication in the liver tumor model are comparable to those of chronically infected human liver. As a practical validation of this mouse model, we showed that IFN-α treatment inhibited HCV RNA replication and abolished GFP expression in the subcutaneous HCC xenografts within 2 wk, which suggests that the mouse model can be used to test the success of other antiviral

strategies against HCV. We showed that IFN-α completely inhibited viral replication in the liver tumor model, and HCV RNA in liver tumors was undetectable by using a highly sensitive RT-nested PCR. The sensitivity of this RT-nested PCR assay was found to be in the range of 1-10 copies of HCV RNA[32]. Using this mouse model, we showed that IFN-α administration completely eliminated HCV sub-genomic RNA replication, which supports the practical use of this animal model to test other antiviral strategies against the background of a growing tumor. This mouse model, therefore, can be used practically to optimize methods for targeted delivery of siRNA and recombinant antibodies that are designed to inhibit HCV replication in tumor xenografts in the mouse liver.

The SCID mouse model described here can be adapted to any research laboratory because it is relatively simple, safe and less expensive, thus allowing it to be used in large numbers for testing. This mouse model can be utilized to establish the success of targeted liver delivery methods of a variety of antiviral strategies within a short period of time. *In vivo* experiments that address the toxicity of different antiviral approaches with this animal model can be tested using a large number of mice to achieve statistical power of significance. This model deals with non-infectious HCV and does not depend on the use of donated or harvested human hepatocytes or infected serum samples. We have also developed *in vivo* adapted Huh-7 cells by elimination of HCV replicon replication. Tumor xenografts that use this Huh-7 cell line in SCID mice can be used to study the infectivity of JFH1 virus and other relevant clinical HCV strains. This will allow us to test the efficacy of neutralizing monoclonal antibodies directed against the HCV envelope protein. There are also some limitations in the tumor xenograft mouse models for HCV that are described here. For example, this model cannot be used for investigation of immunopathogenesis of chronic HCV infection or vaccine development because the SCID mice have defective B- and T-cell responses. Nevertheless, this animal model will be useful for antiviral evaluation and testing the potential of intracellular delivery of siRNA and recombinant antibodies that are directed at inhibition of HCV replication in the liver.

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COMMENTS

Background

Hepatitis C virus (HCV) is the most common blood-borne infection that affects the liver. Several antiviral strategies have been developed during the past 2 years, including small molecules, antisense oligonucleotides, siRNA, ribozymes, and recombinant antibodies that are designed to inhibit HCV replication in cell culture models. A small animal model for HCV is required to test different experimental therapies developed using HCV cell culture systems.

Research frontiers

Development of a small animal model for studying HCV infection has been difficult because the virus only infects humans and chimpanzees. The only available animal model is a chimeric humanized mouse model. This model is very costly, technically very challenging, and very expensive. The authors developed an hepatocellular carcinoma (HCC) xenograft tumor model in SCID mice using a replicon cell line that replicates highly efficient JFH1 virus. The replicon cells were transfected with a green fluorescent protein (GFP)-tagged HCV sub-genomic clone so that the antiviral effect could be observed by GFP expression. The replication of HCV could be inhibited by interferon-α (IFN-α), which indicates that the model can be used for antiviral screening. The HCC xenograft mouse model is less expensive, non-infectious, and can be easily adapted to any research laboratory.

Innovations and breakthroughs

The authors developed an S3-GFP replicon Huh-7 cell line that was highly adapted to mice and formed human HCC xenografts in SCID mice. We showed that HCV replicated in the HCC xenografts formed subcutaneously and in the liver. The antiviral effect of IFN-α showed clearance of HCV in this mouse model.

Applications

This model can be used to optimize methods for liver-targeted delivery of several antiviral strategies against HCV replication.

Peer review

The paper is well written and the scientific content is worth publishing.

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ORIGINAL ARTICLE

Octreotide induces caspase activation and apoptosis in human hepatoma HepG2 cells

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Abstract

AIM: To investigate the role of octreotide on cellular proliferation and apoptosis of human hepatoma (HepG2) cells.

METHODS: We studied cellular proliferation, apoptosis and the possible internal caspase-mediated apoptosis pathway involved, after treatment of HepG2 carcinoma cells with octreotide in comparison with the apoptosis caused by tumor necrosis factor-α (TNF-α). Activities of caspase-3, caspase-9, caspase-8 and caspase-2 were studied, while apoptosis was investigated through detection of DNA fragmentation and through identification of apoptotic cells with the annexin-V/propidium iodide flow cytometric method.

RESULTS: After an initial increase in HepG2 cellular proliferation, a significant inhibition was observed with 10^{-8} mol/L octreotide, while TNF-α dose-dependently decreased proliferation. Early and late apoptosis was significantly increased with both substances. Octreotide significantly increased caspase-3, caspase-8 and caspase-2 activity. TNF-α significantly increased only caspase-2. Cellular proliferation was decreased after treatment with octreotide or TNF-α alone but, in contrast to TNF-α, octreotide decreased proliferation only at concentrations of 10^{-8} mol/L, while lower concentrations increased proliferation.

CONCLUSION: Our findings are suggestive of caspase-mediated signaling pathways of octreotide antitumor activity in HepG2 cells, and indicate that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations.

Key words: Octreotide; Hepatocellular carcinoma; Apoptosis; Caspases; Somatostatin

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause approximately half a million deaths annually[1]. There are striking differences in the incidence of HCC related to

age, gender, race, and geographic region, with hepatitis C virus (HCV) infection acquired 2-4 decades previously explaining at least half of the observed increase in HCC[2]. The survival rates remain generally dismal (median 8 mo)[2]. Undoubtedly, the best available treatment for all liver tumors is complete surgical resection. However, the synthetic somatostatin analogue octreotide has been found effective in inhibiting tumor growth in a variety of experimental models[3,4]. Octreotide did not influence hepatic or portal blood flow, although significantly increased reticuloendothelial system activity[5-7]. Apart from stimulation of the reticuloendothelial system, octreotide may have other mechanisms of action, to inhibit the growth of hepatic tumors. One of the mechanisms suggested may be a direct antiproliferative effect, through receptor-mediated growth inhibition[8]. *In vitro* studies have shown that octreotide demonstrates high affinity binding towards somatostatin receptors sstr2, sstr3 and sstr5, while no binding affinity is found towards receptors sstrl and sstr4[9-11]. It has been reported that octreotide inhibits the proliferation and induces apoptosis of different HCC cell lines *in vitro*[12-18]. The mechanisms of apoptosis induction however are not well understood.

Tumor necrosis factor-α (TNF-α) is a well established as a means of apoptosis induction in a variety of cell types through specific responsive receptors, but other cells require transcriptional arrest[19]. Hepatoma cells treated with TNF-α and cycloheximide (CHX) undergo apoptosis, which is preceded by a strong activation of c-jun N-terminal kinase[20]. The human HCC cell line, SMMC-7721, was insensitive to TNF-α cytotoxicity, but quickly underwent apoptosis in the presence of TNF-α and CHX[21].

Apoptosis is a complex process characterized by caspase activation, cell shrinkage, chromatin condensation and internucleosomal DNA fragmentation[22-25]. Caspases are cysteine-containing aspartic acid-specific proteases and all have similar site-specific proteolytic activity. Caspases are divided into 3 distinct groups based on their substrate specificities. Group I (YVADase) includes caspase-1, -4 and -5 which are involved in cytokine production. Group Ⅱ (DEVDase) caspases, e.g. caspase-3 and -7 are the main effector caspases during apoptosis. These are cleaved by group Ⅲ (IETDase) caspases (e.g. caspase-6, -8, -9 or -10) early in the onset of apoptosis[26]. Upon activation, group Ⅱ caspases act on various cell proteins[26]. Most, but not all, events in apoptosis appear to require a caspase-mediated proteolytic step[25].

Octreotide has been clinically used for treatment of HCC, with conflicting results. Both increased survival[27,28] and no effect[29,30] have been reported. Although negative studies have been criticized[31], the mechanisms by which octreotide may act have not been adequately clarified. Apoptosis may be a fundamental mechanism. In this study, we examined the effect of octreotide on cellular proliferation, apoptosis and caspase activation in HepG2 HCC cells. The model of TNF-α-induced apoptosis was chosen for comparison with octreotide in a study of the biological behavior of caspases, after treatment of HepG2 cells with octreotide.

MATERIALS AND METHODS

Octreotide was from Novartis (Basel, Switzerland) and was used at concentrations of 10^{-10} mol/L to 10^{-7} mol/L, to identify the optimal concentration for inhibition of cell proliferation. Incubations with TNF-α (R&D Systems, Minneapolis, USA) were made at concentrations from 0.1 to 100 ng/mL (0.1, 1, 10, 20, 100 ng/mL). According to the proliferation curve, the suitable concentrations for further experiments were 10^{-8} mol/L for octreotide and 20 ng/mL for TNF-α. These concentrations were used for all further combinations and measurements of apoptotic features.

Cell culture and incubation conditions

The HepG2 cell line is a human hepatocyte carcinoma cell line derived from a well-differentiated human hepatoblastoma and was purchased from the European Collection of Cell Cultures (ECACC, Porton Down, UK). HepG2 cells are maintained in continuous culture in our laboratory in RPMI supplemented with 10% fetal bovine serum (FBS, Gibco, Paisey, UK), at 37℃ and in an atmosphere of 5% CO_2. For experiments, HepG2 cells were seeded in 24-well plates at a density of $2 \times 10^4/cm^2$. Twenty four hours before the experiment, they were cultured in fresh medium without FBS, and then treated with different concentrations and combinations of all substances. Incubations were made at 37℃ in 5% CO_2. Supernatants were collected and stored in -80℃, while cell extracts where used for measurements of caspase activity. Control medium was complete media with 10% FBS. HepG2 cells cultured in control medium are referred to as control cells.

Proliferation assays

For measurement of growth inhibition, the sulforhodamine B colorimetric assay (SRB Assay; Biotium Inc., Hayward, CA, USA) was used, as previously described[32,33]. HepG2 cells were plated in 96-well plates, at an initial density of 5×10^3 cells, with 200 μL medium per well. All substances were added to cultures 1 d after seeding, in order to obtain best attachment of the cells at the beginning of the experiments. Cells were grown for a total of 6 d, with a change of medium and substances on the third day after treatment. Measurements were made as described in the original protocol. Briefly, 50 μL of 50% trichloroacetic acid were placed into the 200 μL medium and plates were stored at 4℃ for 30 min. After washing 5 times with deionized water, plates were left to dry for 24 h at room temperature. Then, 70 μL of 0.4% sulforhodamine B in 1% acetic acid were placed in every well and left at room temperature for 20 min. Before air drying for a second time, plates were washed 5 times with 1% acetic acid. At the end of the procedure, 200 μL of unbuffered Tris-base solution (pH 10.5) were added to each well and measurements were made at 490 nm, subtracting the background at 620 nm. The mean of the optical densities of 8 different controls was considered to be 100% and all other values were expressed as a percentage of the controls.

Detection of apoptosis

DNA fragmentation: For detection of apoptosis, a sandwich, one step, colorimetric enzyme-linked immunosorbent assay, the Cell Death Detection ELISA Plus kit (Roche Diagnostics, Mannheim, Germany) was used. The assay allows for the specific determination of histone-complexed DNA fragments (mono and oligonucleosomes) from the cytoplasm of cells, after the induction of apoptosis. Briefly, after induction of apoptosis and 24-h incubation, the cells were pelleted by centrifugation (200 *g*, 10 min) and the supernatants (containing DNA from necrotic cells that leaked through the membrane during incubation) were discarded. Cells were resuspended and incubated for 30 min in lysis buffer. After lysis, intact nuclei were pelleted by centrifugation. Aliquots of the supernatants were transferred to a streptavidin-coated well of a microtiter plate with 2 monoclonal antibodies, antihistone (biotin-labeled) and anti-DNA (peroxidase-conjugated), so that nucleosomes in the supernatant created antibody-nucleosome complexes, which were continuously bound to the microtiter plate by the streptavidin. All samples were then incubated with peroxidase substrate and absorbance was measured at 405 nm. The mean of the optical densities of 8 different controls was considered to be 100% and all other values were expressed as a percentage of the controls.

Annexin-V/propidium iodide staining: For better evaluation of apoptotic features, the modified Annexin-V Apoptosis Detection Kit (BioVision, Mountain View, CA, US) was used, which is based on the observation that soon after initiation of apoptosis, cells translocate the membrane phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface, but they also shrink, increasing their side scatter (SS) and reducing their forward scatter (FS) characteristics. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin-V, that has a high affinity for PS. Cells that have bound Annexin-V-fluorescein isothiocyanate (FITC) (early apoptotic) show green staining in the plasma membrane, while cells that have lost membrane integrity will show red staining [propidium iodide (PI)] throughout the nucleus and a halo of green staining (FITC) on the cell surface (late apoptotic or necrotic cells).

After treatment and 24-h incubation in 24-well plates, adherent HepG2 cells were gently trypsinized and washed once with serum-containing medium. Then, 1-5 × 10^5 cells were collected by centrifugation (98 *g*, 5 min) and resuspended in 300 μL of 1 × Binding Buffer. After gentle pipetting to resuspend the cell pellets, 3 μL of Annexin-V-FITC and 3 μL of 50 μg/mL PI, were added, followed by a 5-min incubation at room temperature in the dark. Annexin-V-FITC binding was analyzed by flow cytometry (Epics Elite) (Ex = 488 nm; Em = 530 nm) using a FITC signal detector and PI staining by the phycoerythrin emission signal detector. Debris was excluded by scatter gating (forward *vs* side). At least 10000 events were counted for each sample.

Caspase activity

The activities of caspase-3, caspase-9, caspase-8 and caspase-2 were measured. For the evaluation of caspase activity, colorimetric activity assay kits (Chemicon, Temecula, CA, USA) were used. The assays are based on spectophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA (caspase-3), LEHD-*p*NA (caspase-9), IETD-*p*NA (caspase-8) and VDVAD-*p*NA (caspase-2), respectively. Briefly, after treatment and 24-h incubation, supernatants were collected and cells were resuspended in 250 μL of chilled lysis buffer and incubated on ice for at least 10 min. After centrifugation (5 min, 10000 *g*), supernatants (cytosolic extracts) were transferred to a fresh tube and placed on ice. The protein concentration for each sample set was assayed with BIORAD Protein assay (BIORAD, Munchen, Germany)[34]. Samples were incubated for 2-3 h at 37℃ and measured at 405 nm, as indicated. The absorbance of *p*NA from every sample was compared with the uninduced controls and values were expressed as μmol/L of *p*NA per microgram of cytosolic protein (μmol/L per microgram).

Statistics

Statistical analysis was performed using Microsoft Excel 2007 and Instat software (GraphPad software inc., San Diego, California, USA). Results are expressed as mean ± standard error of the mean (SE). The Kolmogorov and Smirnov test was used to check the Gaussian distribution of data. Statistical comparisons were performed using one-way analysis of variance with Tukey's *post hoc* comparisons. The non parametric Kruskal-Wallis test was used instead if Bartlett's test indicated a significant difference between standard deviations. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of octreotide on HepG2 proliferation

Octreotide caused an initial increase in HepG2 proliferation (165.2% ± 6.2% and 127% ± 3% with octreotide 10^{-10} mol/L and 10^{-9} mol/L, respectively) followed by a significant inhibition at a concentration of 10^{-8} mol/L (concentration expected in the blood of patients receiving treatment), reducing cellular proliferation to 77.5% ± 1.9% of control (Figure 1). No difference was observed at a concentration of 10^{-7} mol/L (108.3% ± 3.8%).

The effect of TNF-α was also examined, at concentrations from 0.1 ng/mL to 100 ng/mL. At the low concentrations of 0.1 and 1 ng/mL, TNF-α had no significant effect on the proliferation of HepG2 cells (96.9% ± 5% and 90.6% ± 4.5%, respectively), after 6 d of incubation. A marked inhibitory effect was detected with 10, 20 and 100 ng/mL TNF-α, which reduced cellular proliferation to 69.4% ± 4%, 69.6% ± 2.3% and 61.4% ± 1.7% of control, respectively (Figure 2). However, because TNF-α at a concentration of 20 ng/mL had the optimum inhibitory effect in another HCC cell line (SMMC-7721 cells)[19], this concentration was selected for further experiments.

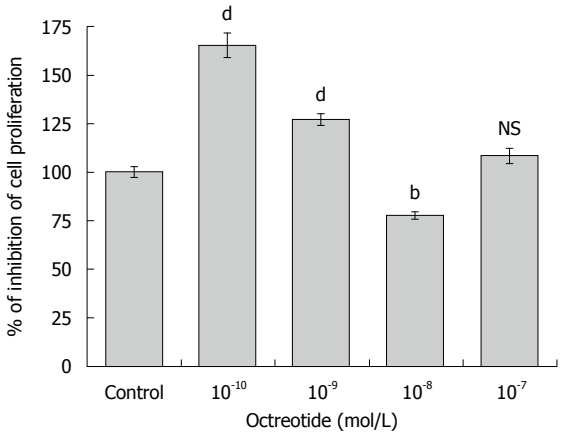

Figure 1 Octreotide at a concentration of 10^{-8} mol/L had a statistically significant inhibitory effect on cellular proliferation of HepG2 hepatocellular carcinoma cells, compared to untreated cells. Lower concentrations caused an initial increase in proliferation. The results represent the mean of 8 different experiments ± SE ($^{b}P < 0.01$, $^{d}P < 0.001$). NS: Not significant.

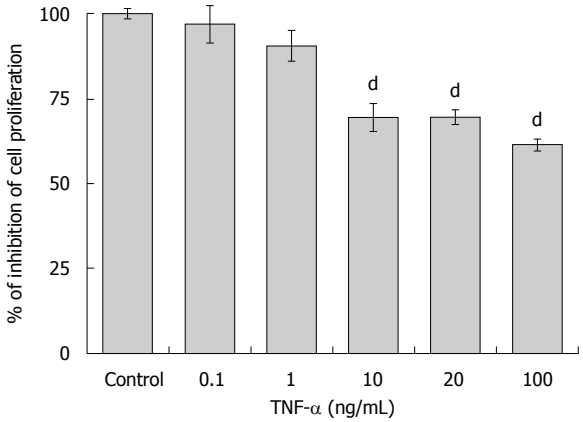

Figure 2 Tumor necrosis factor-α at concentrations of 10, 20 and 100 ng/mL had a statistically significant inhibitory effect on cellular proliferation of HepG2 cells, compared to untreated cells. The results represent the mean of 8 different experiments ± SE ($^{d}P < 0.001$). TNF-α: Tumor necrosis factor-α.

Effect of octreotide on HepG2 apoptosis

Apoptosis was detected based on determination of histone-complexed DNA fragments (mono and oligonucleosomes) from the cytoplasm of apoptotic cells. Similar non significant detection of DNA fragmentation was noted after 24-h treatment of HepG2 cells with either octreotide or TNF-α (115.2% ± 6.95% and 115.2% ± 8.17%, respectively) (Figure 3).

Necrotic cells should be visualized as double positive cells of large dimensions, as they rapidly lose membrane integrity and swelling occurs before destruction, or as fractured membranes of low FS and SS, PI only positive or double positive, but in the place where debris is usually detected and excluded. We also observed that all double positive cells had very small dimensions (data not shown), although they were still intact. We considered double positive cells as cells that followed the apoptotic rather than the necrotic procedure, having increased their SS and decreased their FS characteristics, but still retaining relatively small dimensions. That was the reason why we followed

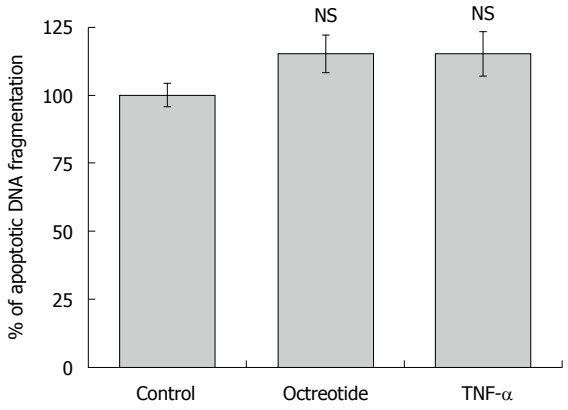

Figure 3 Detection of DNA fragmentation revealed a non significant increase in DNA fragments, after 24-h treatment with octreotide or tumor necrosis factor-α ($n = 8$). TNF-α: Tumor necrosis factor-α; NS: Not significant.

a specific gating strategy for analysis. Octreotide caused a significant increase in early apoptosis (7.2% ± 1.4%, $P < 0.01$, Annexin-V positive cells) and a highly significant increase in late apoptosis (15.3% ± 2.7%, $P < 0.001$, Annexin-V/PI double positive cells). TNF-α significantly increased early (12.5% ± 1.4%, $P < 0.001$) and more so late apoptosis (26.4% ± 4%, $P < 0.001$, Figure 4). All comparisons were made with untreated HepG2 cells used as control cells (0.5% ± 0.3% and 2 ± 0.3% for early and late apoptosis, respectively) (Figure 4).

Effect of octreotide on caspase activity in HepG2 cells

Caspase-3 activity was significantly increased after treatment of HepG2 cells with octreotide (4.71 ± 0.81 μmol/L per microgram protein, $P < 0.01$) alone, while after TNF-α only, a non significant increase was found (3.28 ± 0.55 μmol/L per microgram protein), compared with uninduced cells (1.87 ± 0.24 μmol/L per microgram protein) (Figure 5). A small but not significant increase in caspase-9 activity was detected after treatment of HepG2 cells with TNF-α (2.44 ± 0.33 μmol/L per microgram protein) or octreotide alone (2.42 ± 0.77 μmol/L per microgram protein) (Figure 5), compared to uninduced cells (1.56 ± 0.21 μmol/L per microgram protein). TNF-α caused a non significant increase in caspase-8 activity (0.9 ± 0.18 μmol/L per microgram protein) compared with control cells (0.51 ± 0.06 μmol/L per microgram protein) (Figure 5). However, octreotide caused a significant increase (1.3 ± 0.1 μmol/L per microgram protein, $P < 0.01$). TNF-α and octreotide caused a significant increase in caspase-2 activity (1.73 ± 0.17 μmol/L per microgram protein and 1.7 ± 0.18 μmol/L per microgram protein, respectively, $P < 0.001$), compared with control cells (0.8 ± 0.09 μmol/L per microgram protein) (Figure 5).

DISCUSSION

Clinical studies of non-neuroendocrine tumors demonstrate that octreotide can inhibit the growth of a variety of tumors, either directly, through binding on the sstrs of tumor cells, or indirectly, through an immunomodulatory or an antiangiogenic effect[35-37]. Several reports indicate that

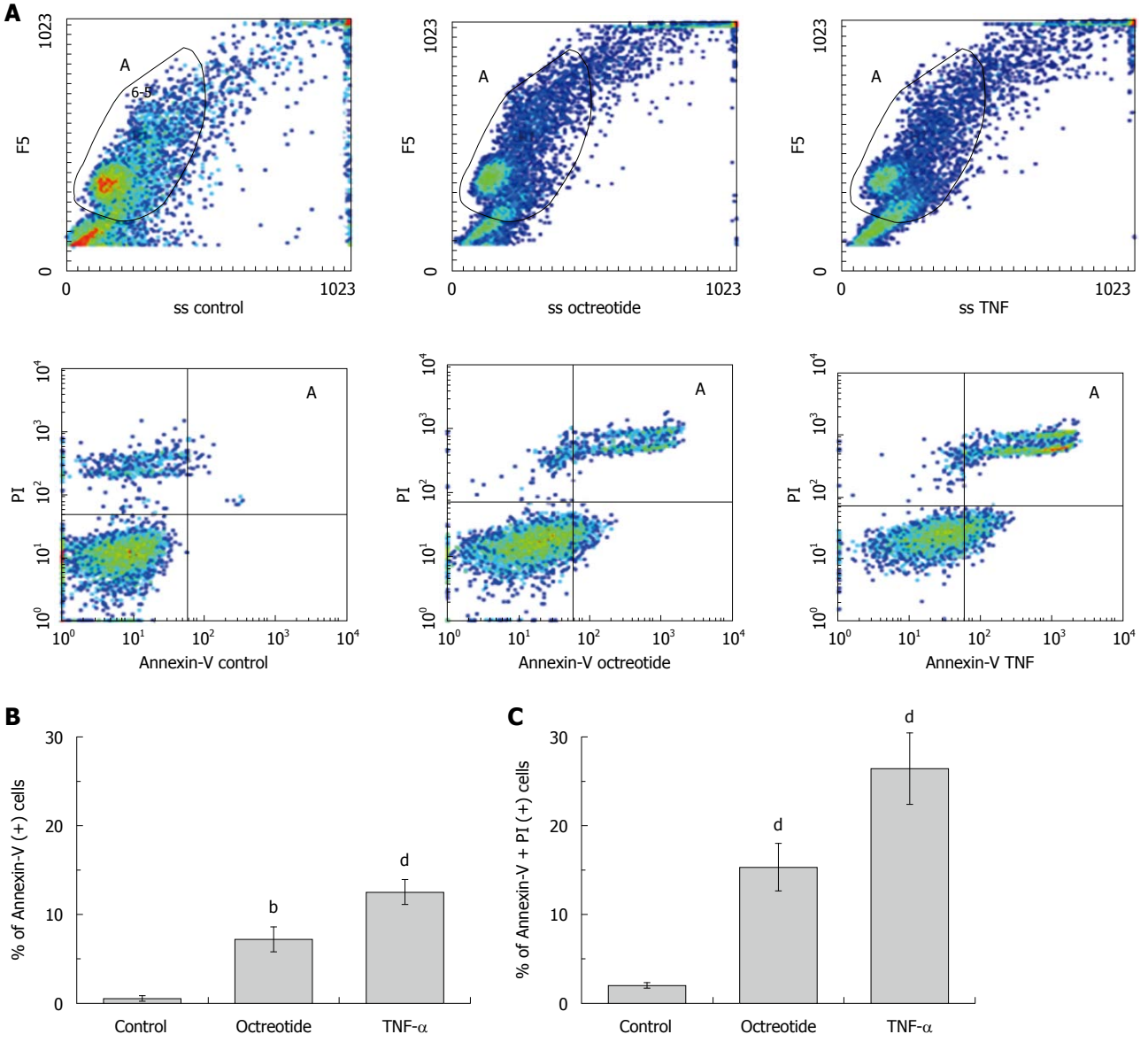

Figure 4 The apoptotic effect of octreotide and tumor necrosis factor-α alone is shown. A: Octreotide and tumor necrosis factor-α (TNF-α) significantly increased early (Annexin-V only positive, right lower quadrant) and more so late apoptotic cells [Annexin-V and propidium iodide (PI) positive, right upper quadrant]. Every sample was analyzed with the same gating strategy (Gate A) to exclude debris and non-specific binding of Annexin-V, while control refers to uninduced HepG2 cells; B: Annexin-V positive cells; C: Annexin-V/PI positive cells. B and C: Mean of 8 different experiments ± SE ($^bP < 0.01$, $^dP < 0.001$).

octreotide inhibits the proliferation and induces apoptosis of HCC cells *in vitro*[12-18]. In this study we confirmed that octreotide inhibits HepG2 proliferation[12,15,18], but only at a concentration of 10^{-8} mol/L, although an initial increase at lower concentrations was observed.

In contrast to these findings, there are also reports that proliferation of HCC cells or hepatic stellate cells is not affected by octreotide[38,39]. In the study of Reynaert *et al*[39], shorter periods of culture compared to ours were used, while activation of sstrs was achieved with individual synthetic agonists, therefore a possible combined effect of concomitant receptor activation may have been missed. Similarly, clinical trials have demonstrated a survival benefit of patients with inoperable HCC treated with octreotide[27,28], but also negative studies have been published[29,30] and recently criticized[31]. Interestingly, in our study, lower concentrations of octreotide increase proliferation and this is possibly an additional reason for divergent results in both clinical trials and *in vitro* studies of octreotide in HCC. Our findings also indicate that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations.

Octreotide binds mainly to sstr2, sstr3 and sstr5[40], the presence of which has been recently documented in HepG2 cells[12,41]. The antiproliferative effect of octreotide is thought to be mediated by sstr2[42] and sstr5[43]. Even when a significant amount of sstr2 binding in cellular membranes is not evident, it is possible that octreotide is internalized either along with sstr2 or alone as reported by Dournaud *et al*[44] and Hornick *et al*[45]. Recently a desensitization of sstr2 has been reported after short term incubation of an HCC cell line with octreotide, which is probably reversed after long term incubation[46]. We have previously reported an IC_{50} of 1.25 nmol/L for the

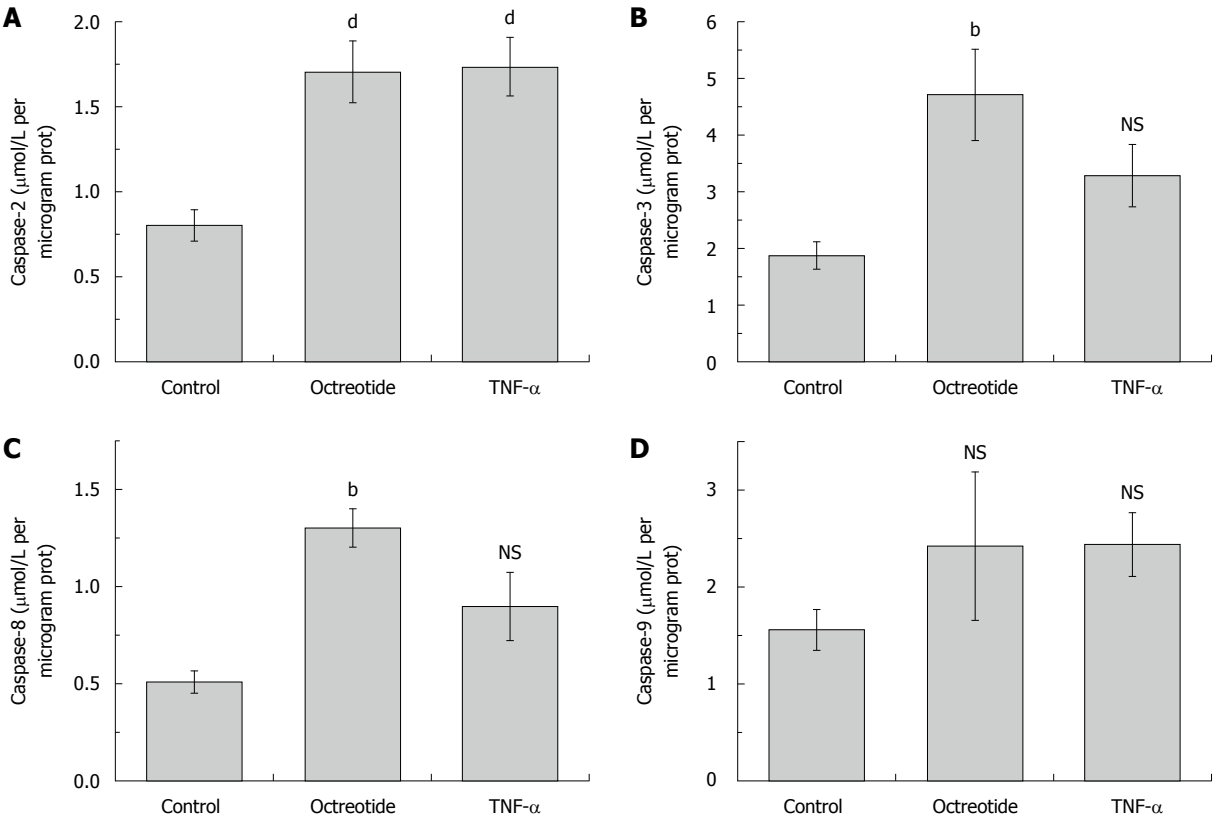

Figure 5 Caspase-2 (A), -3 (B), -8 (C) and -9 (D) activities, after treatment of HepG2 cells with 10^{-8} mol/L octreotide and 20 ng/mL tumor necrosis factor-α, compared to untreated cells. The results represent the mean of 10 different experiments ± SE ($^{b}P < 0.01$, $^{d}P < 0.001$). TNF-α: Tumor necrosis factor-α; NS: Not significant.

antiproliferative effect of octreotide for HepG2 cells[41] which is within the range of IC_{50} for sstr2 but it is lower from the IC_{50} reported for sstr5. In the case of sstr5, it is possible that a biological effect can be achieved without activation of the total number of receptors.

The antiproliferative effect of octreotide may be due to either cell necrosis or cell apoptosis[47]. Therefore, we investigated the apoptotic effect of octreotide, particularly in association with caspase activation, comparing this effect with the well-described pathways of TNF-α-mediated apoptosis. The machinery of apoptosis includes death receptors, adaptor proteins and proteolytic enzymes (caspases). Death receptors belong to the tumor necrosis factor receptor gene superfamily. Among these receptors, TNF receptor-1 (TNFR1) and Fas (CD95) are the most extensively characterized, and both are abundantly expressed in liver[20,48]. TNF-α at 20 ng/mL induced apoptosis in human hepatoma cell line SMMC-7721 *in vitro*, which was exacerbated by the hypoxanthine-xanthine oxidase system and CHX, but alleviated by superoxide dismutase, suggesting that TNF-α-induced apoptosis may be due to oxidative stress[49]. The SMMC-7721 cell line was insensitive to TNF-α cytotoxicity and underwent apoptosis quickly in the presence of TNF-α and CHX[21]. In accordance with this study, the optimal concentration of TNF-α was also found to be 20 ng/mL for HepG2 cells in our study.

Our findings with flow cytometry showed that both octreotide and TNF-α induced a significant early and late apoptosis of HepG2 cells. DNA fragmentation measurements also demonstrated a non significant induction of apoptosis. This possibly means that flow cytometry is a more sensitive method for quantification of apoptosis.

The mechanism by which octreotide induces apoptosis is not well understood. Changes in sstr expression because of downregulation or possible heterodimerization[27,50] of a receptor, together with changes in the expression of regulatory proteins required for correct trafficking of specific sstr subtypes, could affect the direct antitumor effect of octreotide, which has been previously demonstrated in models expressing sstr2[51,52]. Mediated by sstr2, octreotide upregulates tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), death receptor 4 (DR4) and TNFR1, and downregulates Bcl-2, which results in apoptosis[53]. Mediated by sstr3, octreotide upregulates p53[47] or induces Bcl-2-associated protein Bax[47,54] and acidic endonuclease, resulting in apoptosis. In addition to these mechanisms, in this study we demonstrated a direct effect of octreotide on caspase activation. The effect of octreotide on caspase-mediated apoptosis, is limited to caspase-3 activation, as the main effector caspase supportive of apoptosis and it was detected in primary pheochromocytoma cells[55], in radiation-induced intestinal damage[56] and in activated lymphocytes[57]. Interestingly, decreased caspase-3 mRNA expression in Kupffer cells also indicates a possible additional beneficial effect of octreotide in HCC, through an antiapoptotic effect on Kupffer cells[58].

We suggest a caspase-mediated apoptotic pathway after treatment of HCC cells with octreotide, where, unlike TNF-α induced apoptosis, 3 out of 4 caspases tested were significantly increased. The activation of all caspases

indicates a possible mitochondria-dependent apoptotic pathway. In contrast, findings from TNF-α-induced apoptosis possibly indicate a different pathway.

A TNF-α-mediated pathway is reported to activate caspase-8, which promotes cleavage of various downstream caspases, including caspases-3, -6 and -7. Caspase-8 can also cleave the Bcl-2 homologue Bid to reveal an active truncated Bid fragment inducing cell death through a mitochondrial pathway[59-62]. However in our study, caspase-8, caspase-3 and caspase-9 were all increased by TNF-α, but this was not statistically significant. Furthermore, caspase-2 was significantly increased by TNF-α, a finding not reported before. In a previous study, we presented a TNF-α-induced increase of caspase-2, but this increase did not reach the statistical significance[63]. In the present study, with an increased number of experiments, we found a significant increase in caspase-2 by TNF-α in HepG2 cells. This may be related to inefficient cleavage of Bid, so that all caspases can be significantly activated[59].

Caspase-2 seems to play critical and specific roles in programmed cell death[64]. It has been difficult to assign caspase-2 to the effector or initiator caspase groups. Cytokine-induced and stress-induced apoptosis act through conceptually similar pathways in which mitochondria are amplifiers of caspase activity rather than initiators of caspase activation[65]. In our study, caspase-2 appeared to be activated independent of significant (octreotide) or non-significant (TNF-α) activation of the mitochondria-mediated pathway. This may have been the result of intracellular events (such as pH or stress) or feedback activation by effector caspases (such as caspase-3).

Thus, our findings suggest that in HepG2 cells octreotide probably causes apoptosis by a mitochondrial apoptosis pathway, sequentially implicating caspase-8, -2, -9 and -3, although further experiments are required to define the exact initiator pathway. TNF-α on the other hand seems to induce caspase-2 activation, possibly mediated through oxidative stress, as suggested before[65]. The non-significant activation of the extrinsic pathway (caspase-8) or of the intrinsic pathway (caspase-9), perhaps due to inefficient Bid cleavage, is maybe the cause of the resistance observed in previous studies and of the eliminated TNF-α-mediated apoptotic effects observed in our study.

In summary, our results support the induction of a caspase-mediated apoptotic pathway by octreotide in HCC cells, implicating both the receptor-mediated and the mitochondrial-apoptotic pathway. The correlation of specific apoptotic, caspase-mediated pathways, with the expression of sstrs in HCC cells needs more investigation to better define and clarify the intracellular mechanisms of the antiproliferative effects of octreotide.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause approximately half a million deaths annually. Undoubtedly, the best available treatment for all liver tumors is complete surgical resection. However, the synthetic somatostatin analogue octreotide has been found effective in inhibiting tumor growth in a variety of experimental models.

Research frontiers

Apart from stimulation of reticuloendothelial system, octreotide may have other mechanisms of action, to inhibit the growth of hepatic tumors. It has been reported that octreotide inhibits the proliferation and induces apoptosis of different HCC cell lines *in vitro*. The mechanisms of apoptosis induction however are not well understood.

Innovations and breakthroughs

Several reports indicate that octreotide inhibits the proliferation and induces apoptosis of HCC cells *in vitro*. In this study, the authors confirmed that octreotide inhibited HepG2 proliferation at a concentration of 10^{-8} mol/L. Interestingly, lower concentrations of octreotide increased proliferation and this is possibly an additional reason for divergent results in both clinical trials and *in vitro* studies of octreotide in HCC. Also, their results support the induction of a caspase-mediated apoptotic pathway by octreotide in HepG2 cells, implicating both a receptor-mediated and mitochondrial-apoptotic pathway.

Applications

The findings of the present study indicate that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations. Also, based on the recently documented presence of sstr2, sstr3 and sstr5 in HepG2 cells, the need for further correlation of specific apoptotic, caspase-mediated pathways, with the expression of somatostatin receptors in HCC cells, is highlighted, to better define and clarify the intracellular mechanisms of the antiproliferative effects of octreotide.

Peer review

The authors evaluated the role of octreotide on cellular proliferation and apoptosis of HepG2 cells. Their results support the induction of a caspase-mediated apoptotic pathway by octreotide in HepG2 cells, implicating both a receptor-mediated and a mitochondrial-apoptotic pathway. They, also, indicated that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations.

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ORIGINAL ARTICLE

Effect of heme oxygenase-1 on renal function in rats with liver cirrhosis

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Abstract

AIM: To investigate the role of heme oxygenase-1 (HO-1) in pathogenesis of experimental hepatorenal syndrome (HRS).

METHODS: Rats were divided into liver cirrhotic group, zinc protoporphyrin IX (ZnPP) treatment group, cobalt protoporphyrin (CoPP) treatment group and sham group. Biliary cirrhosis was established by bile duct ligation in the first three groups. Rats in the ZnPP and CoPP treatment groups received intraperitoneal injection of ZnPP and CoPP, respectively, 24 h before sample collection. Expression of HO-1 mRNA in kidney was detected by reverse-transcription polymerase chain reaction, while protein expression was determined by immunohistochemical analysis. Hematoxylin and eosin staining was performed to observe liver cirrhosis and renal structure. Renal artery blood flow, mean arterial pressure and portal vein pressure, 24 h total urinary volume, serum and urine sodium concentrations, and creatinine clearance rate (Ccr) were also measured.

RESULTS: The HO-1 mRNA and protein expression levels in kidney, 24 h total urinary volume, renal artery blood flow, serum and urine sodium concentration and Ccr were lower in cirrhotic group than in sham group ($P < 0.05$). However, they were significantly lower in ZnPP treatment group than in cirrhotic group and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$).

CONCLUSION: Low HO-1 expression level in kidney is an important factor for experimental HRS.

Key words: Heme oxygenase-1; Carbon monoxide; Hepatorenal syndrome; Zinc protoporphyrin IX; Cobalt protoporphyrin; Bile duct ligation; Biliary cirrhosis

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INTRODUCTION

Renal dysfunction is very common in patients with advanced liver disease or cirrhosis. Its severity ranges from electrolyte-balance disturbances and water retention to hepatorenal syndrome (HRS)[1], which is a unique form of renal failure associated with liver cirrhosis or portal hypertension[2]. Although HRS represents a functional form and sometimes a reversible form of renal failure without significant changes in renal histology during the course of decompensated cirrhosis[3,4], it is a poor prognostic indica-

tor for patients with liver cirrhosis, who show an increased risk of morbidity and mortality[2,3]. So far, no effective strategies are available for the treatment or prevention of HRS. Instead, patients are usually managed by maintaining their adequate hemodynamic status and intravascular volume. A better understanding of the pathophysiological mechanism underlying HRS helps to guide its treatment.

It is currently believed that marked renal vasoconstriction and predominant peripheral arterial vasodilation play a critical role in the pathogenesis of HRS[5-7]. Previous studies have shown that nitric oxide (NO), a potent vasodilator, plays an important role in the development of hyperdynamic syndrome and peripheral vasodilation during cirrhosis[8]. Increased NO level and synthetase activity in patients with liver cirrhosis have adverse effects on the functions of renal tubules and glomeruli[9], and inhibition of NO synthetase prevents the development of renal failure in an animal model of HRS[10,11]. Carbon monoxide (CO), a byproduct of heme oxygenase-1 (HO-1), shares many characteristics with NO. Endogenous CO is an activator of soluble guanylate cyclase and relaxes vascular smooth muscle in a cGMP-dependent or a cGMP-independent manner[12,13]. Studies have shown that the HO-1/CO system plays an important role in the control of vascular tone and that inhibition of HO-1 blocks vasodilation induced by heme[12-14]. HO-1 is also involved in the prevention of renal failure after renal ischemia[15] or glycerol-induced acute renal injury in rats[16].

Metalloporphyrins constitute a class of compounds in which the central iron of heme is replaced by other metals such as cobalt and zinc[17]. These metalloporphyrins inhibit or induce HO-1. This study was to evaluate the expression of HO-1 in kidneys of rats with experimental HRS and the functional role of HO-1 in the pathogenesis of HRS by manipulating its activity *via* intraperitoneal injection of either zinc protoporphyrin IX (ZnPP), a specific HO-1 enzyme inhibitor, or cobalt protoporphyrin (CoPP), a specific HO-1 enzyme inducer.

MATERIALS AND METHODS

Animals

Healthy male Sprague Dawley rats, weighing 200-220 g, were obtained from the Laboratory Animal Center of Dalian Medical University.

Reagents

ZnPP and CoPP (Sigma, St Louis, MO, USA) were dissolved in 0.2 mol/L of NaOH, adjusted to a pH of 7.4 and diluted in 0.85% NaCl with a final concentration of 1 mg/mL as previously described[18]. Rabbit anti-mouse HO-1 antibody (Boster Biological Technology, Wuhan, China), anti-rabbit IgG (MaxVisionTM2, Maixin Biotechnology, Fuzhou, China), TaKaRa RNA polymerase chain reaction (PCR) kit (AMV) Version 3.0 (TaKaRa Biotechnology, Dalian, China) were used in the study.

Animal model and grouping

The rats were randomly divided into sham group (n = 8), cirrhotic group (n = 10), ZnPP treatment group (n = 9) and CoPP treatment group (n = 8). They were well fed and housed for 3 d before any experimental protocols. Biliary cirrhosis was induced by bile duct ligation (BDL)[19,20] in rats of the cirrhotic group, ZnPP and CoPP treatment groups. The surgical procedures were approved by the Animal Care and Use Committee of Dalian Medical University. Laparotomy was performed under anesthesia with ether. The bile duct was isolated and double-ligated with a 3-0 silk suture. The abdominal wall and skin were closed with a 4-0 silk suture, and the antibiotic benzathine benzylpenicillin powder was sprinkled over the closed incision. The rats were continuously fed and housed for a further 4-wk period after surgery, and samples were collected. Rats in sham group underwent laparotomy with the bile duct isolated but not ligated. Rats in ZnPP and CoPP treatment groups received an intraperitoneal injection with ZnPP[21] or CoPP (30 mg/kg body weight) once, 24 h before sample collection. Rats in the 4 groups were housed in metabolic cages for the last 24 h, and urine was collected to measure its volume and the sodium and creatinine (Cre) levels.

Sample collection

Four weeks after surgery, the rats were anesthetized with ether and their portal vein, right carotid artery, and renal artery were isolated. Renal artery blood flow was measured by ultrasound (LOGIQ7, GE, USA). A catheter, connected to a pressure transducer (BL-420F biological experimental system, Chengdu Technology and Market Co. Ltd., China), was placed in the carotid artery for measurement of mean arterial pressure (MAP), then 1 mL of arterial blood was collected in a heparinized syringe through an arterial catheter to measure carboxyhemoglobin (COHb) using a RapidLab 1245 blood gas analyzer (Siemens, USA), as an index for the CO level in arterial blood. The catheter was placed in the portal vein to measure portal vein pressure (PVP). Then, 4 mL of blood was collected from the rats to measure serum levels of bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), Cre and sodium with a Hitachi 7600-110 automatic biochemical analyzer (Hitachi Co., Tokyo, Japan). Urine levels of Cre and sodium were also measured using this machine. The Cre clearance rate (Ccr) was calculated as urine Cre × urine volume/serum Cre. The left kidney and one liver lobe were excised, some of their tissues were fixed in a 10% neutral formalin solution and embedded in paraffin, while the remaining tissues were preserved at -80℃ for PCR.

Reverse-transcription PCR analysis

Total RNA was extracted from kidneys following a standard guanidinium phenol-chloroform extraction protocol. The quantity of RNA was determined by measuring the optical density at 260 nm ($A_{260\ nm}$ = 1 for 40 μg/mL RNA), and the purity of RNA was assessed by determining the ratio of the optical density obtained at 260 and 280 nm (pure RNA: $A_{260\ nm}/A_{280\ nm}$ = 2.0) using a Shimadzu UV-1206 spectrophotometer (Shimadzu, Japan). The primer sequences for HO-1 are 5'-ACTTTCAGAAGGGTCAGGTGTCC-3' (forward) and 5'-TTGAGCAGGAAGGCGGTCTTAG-3'

Table 1 Effect of bile duct ligation and cobalt protoporphyrin or zinc protoporphyrin IX treatment on serum and urine levels of creatinine and sodium, and creatinine clearance rate (mean ± SD, *n* = 8-10 per group)

	Sham group	Cirrhosis group	CoPP group	ZnPP group
Serum Cre (μmol/L)	30.4 ± 1.81	36.3 ± 6.27[a]	33.5 ± 5.98	45.3 ± 8.92[c]
Urine Cre (μmol/L)	7.18 ± 1.15	8.08 ± 2.50	4.49 ± 1.51	6.31 ± 1.20
Serum sodium (mmol/L)	142.86 ± 3.44	138.75 ± 0.96[a]	142.64 ± 5.43[c]	136.57 ± 1.40[c]
Urine sodium (mmol/L)	91.50 ± 12.12	71.33 ± 10.07[a]	109.15 ± 64.93	66.25 ± 11.8
Ccr (mL/min)	0.23 ± 0.02	0.12 ± 0.05[a]	0.14 ± 0.04	0.07 ± 0.01[c]
AST (IU/L)	156.8 ± 18.28	237.2 ± 95.13[a]	467.14 ± 222.28[c]	209.11 ± 65.77

[a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* cirrhotic group. CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin IX; Cre: Creatinine; Ccr: Creatinine clearance rate; AST: Aspartate aminotransferase.

(reverse) and the product size is 524 bp, while the primer sequences for β-actin are 5'-GGAGTCAACGGATTTGGT-3' (forward), 5'-GTGATGGGATTTCCATTG-3' (reverse) and the product size is 226 bp. An aliquot of each mixture was used for reverse-transcription (RT)-PCR amplification using reagents purchased from Takara Bio Inc (Dalian, China). PCR products were separated by 2.5% agarose gel electrophoresis. The product bands were photographed and the density of each product band was quantified. The results were expressed as the ratio of the band density for HO-1 mRNA to that of β-actin mRNA.

Immunohistochemical analysis

Kidney and liver tissues were fixed in a 10% neutral formalin solution and embedded in paraffin wax and cut into sections. Some sections were routinely stained with HE while the other sections underwent deparaffinization, rehydration and inactivation, and were incubated with rabbit-anti-mouse HO-1 monoclonal antibody (1:50) at room temperature for 60 min, and then with secondary antibody (MaxVisionTM2) at room temperature for 15 min. The sections were mounted after staining. The primary antibody was replaced by phosphate-buffered saline to serve as a negative control. Five high-power microscopic fields were randomly chosen per slide and the yellow material in cytoplasm was considered to represent a HO-1-positive cell. Cell staining was assigned to 4 scores: 4 = > 75% positive cells, 3 = 50%-75% positive cells, 2 = 25%-50% positive cells, and 1 = < 25% positive cells. Cell staining intensity was scored based on its color as follows: 0 = no staining, 1 = faint yellow, 2 = light brown, and 3 = dark brown[22]. The final score was defined as staining intensity × percentage of positive cells. The mean score of five fields was used to compare the four groups.

Statistical analysis

Data analysis was performed using the SPSS 10.0 software (Chicago, IL, USA). Analysis of variance or Wilcoxon statistical methods were used to determine statistical significance. All measurements in this study were expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Biochemical examination

The serum level of AST in biliary cirrhotic group was 237.2 ± 95.13 IU/L, which was significantly higher than that (156.8 ± 18.28 IU/L) in sham group ($P < 0.05$). The serum level of Cre was significantly higher and the Ccr was significantly lower in biliary cirrhotic group than in sham group ($P < 0.05$). The serum Cre level was significantly higher in ZnPP treatment group ($P < 0.05$) and slightly lower in CoPP treatment group than in cirrhotic group ($P > 0.05$). The serum and urine sodium levels were significantly lower in cirrhotic group than in sham group ($P < 0.05$). The serum sodium concentration was significantly lower in ZnPP treatment group and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$, Table 1).

Hemodynamic parameters and arterial blood gas levels

The PVP was significantly higher and the MAP was significantly lower in cirrhotic group than in sham group ($P < 0.01$). However, no significant difference was found in PVP and MAP in ZnPP and CoPP treatment groups compared with cirrhotic group. The COHb level in arterial blood was significantly higher in cirrhotic group than in sham group ($P < 0.05$) while significantly lower in ZnPP treatment group and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$). The renal artery blood flow was significantly lower in cirrhotic group than in sham group ($P < 0.01$), while significantly lower in ZnPP treatment group and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$). Furthermore, the 24 h urine volume was significantly smaller in cirrhotic group than in sham group ($P < 0.05$), while significantly smaller in ZnPP treatment group and significantly larger in CoPP treatment group than in cirrhotic group ($P < 0.05$, Table 2).

Histopathological analysis of liver and kidney in cirrhotic and sham rats

Liver and kidney tissue samples from cirrhotic and sham rats were stained with HE to examine the histopathological changes. The bridging necrosis of hepatic cells was observed in livers of rats 4 wk after BDL, particularly in portal areas, nodular regeneration of hepatocytes, collapse, and disorganization of the hepatic lobular structure, numerous lymphocytes infiltrating the portal area and around the central vein, and the formation of pseudolobules surrounded by fibrous septa. In contrast, except for vascular dilation and congestion of the mesenchyme, no

Table 2 Effects of bile duct ligation and cobalt protoporphyrin or zinc protoporphyrin IX treatment on hemodynamic parameters, carboxyhemoglobin and 24-h urine volume (mean ± SD, *n* = 8-10 per group)

	Sham group	Cirrhosis group	CoPP group	ZnPP group
PVP (mmHg)	9.24 ± 0.76	15.56 ± 2.36[b]	17.28 ± 1.20	13.71 ± 1.39
MAP (cmH_2O)	118.83 ± 8.09	59.23 ± 12.19[b]	52.75 ± 5.76	67.76 ± 7.66
COHb (%)	0.23 ± 0.05	0.50 ± 0.20[a]	0.83 ± 0.39[c]	0.23 ± 0.06[c]
RABF(mL/min•100 g)	3.89 ± 0.09	3.58 ± 0.04[b]	3.76 ± 0.06[c]	3.50 ± 0.08[c]
Urine (mL/24 h)	15.00 ± 2.23	10.93 ± 1.92[a]	13.5 ± 1.10[c]	8.50 ± 1.10[c]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* sham group; [c]$P < 0.05$ *vs* cirrhosis group. CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin IX; PVP: Portal vein pressure; MAP: Mean arterial pressure; COHb: Carboxyhemoglobin; RABF: Renal arterial blood flow.

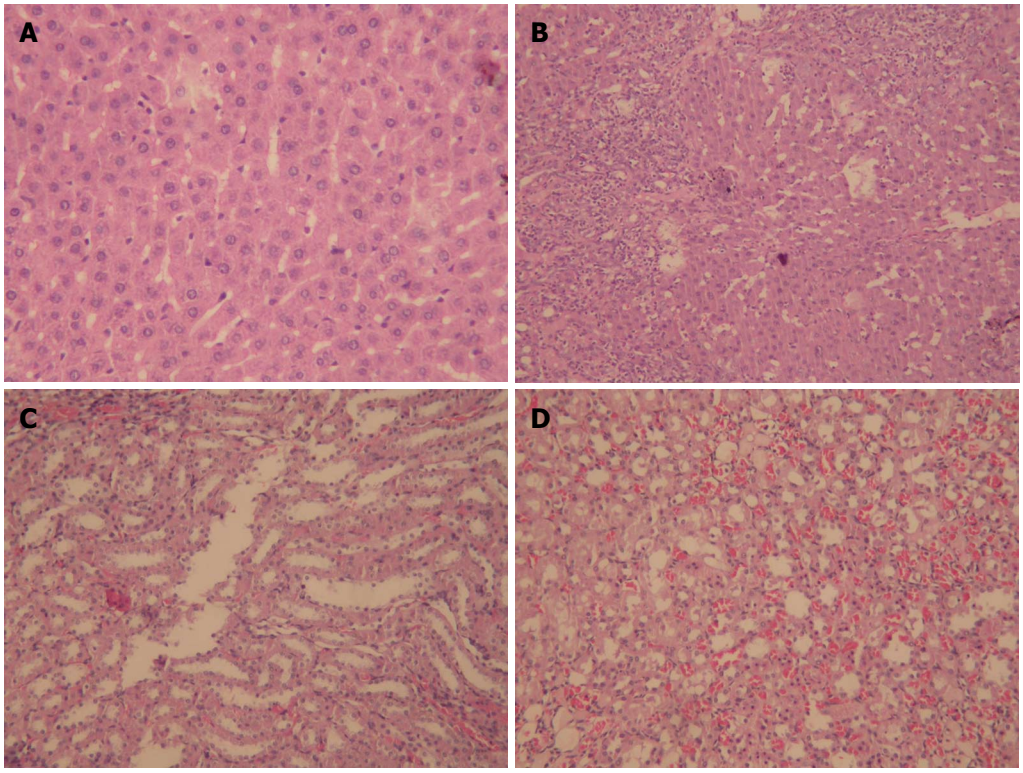

Figure 1 Representative photomicrographs of rats in cirrhotic and sham groups (magnification 200 ×, HE staining). A: Normal liver structure; B: Liver cirrhosis; C: Normal kidney structure; D: Renal structure in cirrhotic group.

obvious pathological changes were observed in kidneys of cirrhotic group compared with sham group (Figure 1).

Renal HO-1 mRNA expression level

RT-PCR showed that the expression level of HO-1 mRNA in kidney was significantly lower in cirrhotic group than in sham group ($P < 0.01$). Furthermore, renal HO-1 mRNA expression was significantly decreased in ZnPP treatment group ($P < 0.05$) and significantly higher in CoPP treatment group than in cirrhotic group ($P < 0.05$) (Figure 2).

Immunohistochemical detection of HO-1 protein in kidney and liver

To localize the HO-1 protein expression in kidneys, immunohistochemistry was performed using specimens from the four groups. The HO-1 protein was mainly expressed in the distal renal tubules, which is similar to reported findings[23] (Figure 3). The intensity and percentage of cells expressing HO-1 protein in kidney were also detected. Mild staining was observed in renal tissue samples from sham group, with a score of 1.21 ± 0.33. The HO-1 score was 0.79 ± 0.25 in cirrhotic group, which was significantly lower than that in sham group ($P < 0.01$). The HO-1 score (0.21 ± 0.25) was lower in ZnPP treatment group and higher in CoPP treatment group (2.46 ± 0.46) than in cirrhotic group ($P < 0.05$). The staining intensity and percentage of cells expressing HO-1 protein were also evaluated in liver of cirrhotic and sham groups. Unlike the HO-1 protein expression in kidney, the mean hepatic HO-1 score was significantly higher in cirrhotic group than in sham group (4.63 ± 0.74 *vs* 0.63 ± 0.52, $P < 0.01$).

DISCUSSION

Renal dysfunction is very common in patients with ad-

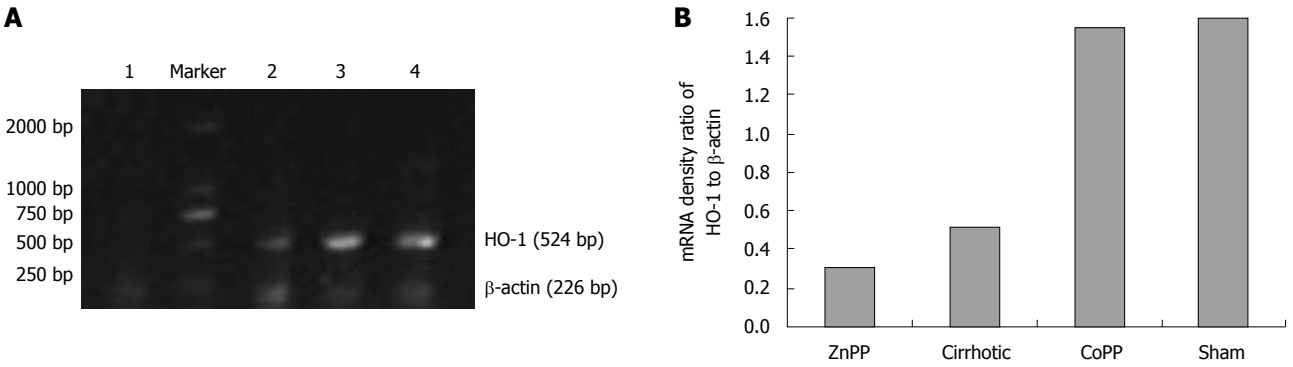

Figure 2 Expression of heme oxygenase-1 mRNA in kidney. A: Representative reverse-transcription polymerase chain reaction data showing the heme oxygenase-1 (HO-1) mRNA expression levels in kidneys from zinc protoporphyrin IX (ZnPP) treatment group (lane 1), cirrhotic group (lane 2), cobalt protoporphyrin (CoPP) treatment group (lane 3), and sham group (lane 4); B: Quantitative data showing the ratio of band density of the corresponding HO-1 mRNA to that of β-actin mRNA.

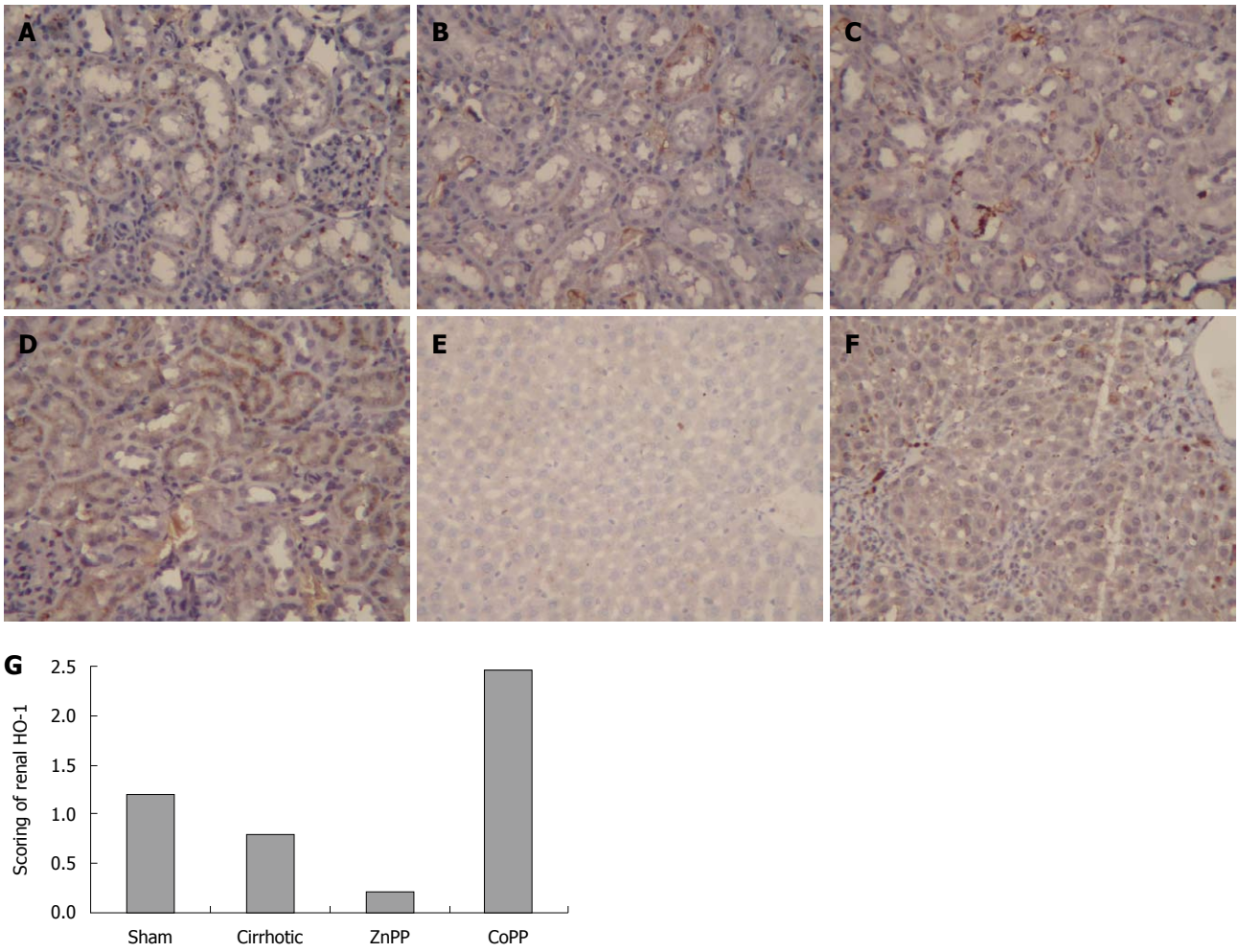

Figure 3 Expression of heme oxygenase-1 protein in kidney and liver. Immunohistochemical staining of renal heme oxygenase-1 (HO-1) protein in rats of sham group (A), cirrhotic group (B), zinc protoporphyrin IX (ZnPP) treatment group (C), cobalt protoporphyrin (CoPP) treatment group (D), and immunohistochemical staining of hepatic HO-1 protein in rats of sham group (E) and cirrhotic group (F) in the upper part (magnification 200 ×), and quantitative scoring (G) of immunohistochemical staining of renal HO-1 protein expression in each group in the lower part.

vanced cirrhosis, and its severity varies from electrolyte-balance disturbances and water retention to HRS. HRS is considered a functional renal failure because no structural damage has been observed in kidney, and can be reversed in some cases[4]. However, it carries a worse prognosis of patients with cirrhosis and increases their risk of mortality[2,3]. It has been reported that the annual incidence of HRS in patients with liver cirrhosis and ascites is about 8%[24]. Typical features of HRS include oliguria, hyponatremia, azotemia, and hyponatruria. Although the pathophysiological mechanism underlying HRS is still incompletely understood, marked renal vasoconstriction in the presence

of splanchnic and systemic vasodilation may play an important role, and may thus reduce the renal arterial blood flow and the glomerular filtration rate, resulting in oliguria and an increased serum Cre concentration[6-8]. Studies have shown that the HO-1/CO system plays an important role in the control of vascular tone and that inhibition of HO-1 blocks vasodilation induced by heme[12-14]. In addition, HO-1 is also involved in the prevention of renal failure after renal ischemia[15] or glycerol-induced acute renal injury in rats [16]. In this study, the relation between expression of HO-1 in kidney and renal arterial blood flow and renal function was investigated in cirrhotic rats.

To evaluate the hepatic, renal, and systemic changes in rats 4 wk after BDL, the MAP, PVP, COHb in arterial blood, serum levels of AST and ALT, Cre and sodium, urine sodium and Cre were measured. The changes in hepatic and renal histology were also examined, and the expression levels of HO-1 mRNA and protein in liver and kidney were evaluated. The Ccr level was also measured as an index of glomerular filtration rate and renal function. The serum level of AST was significantly higher in cirrhotic group than in sham group, indicating that BDL causes marked liver injury, with liver cirrhosis confirmed with HE staining of liver specimens. In addition, the MAP level was significantly lower in BDL rats than in time-matched sham rats, indicating that a hyperdynamic state occurs. Furthermore, the PVP was significantly higher in BDL rats than in time-matched sham rats, indicating that portal hypertension exists. Oliguria, hyponatremia, hyponaturia, increased Cre concentrations and decreased Ccr were also observed in BDL rats. All these findings show that experimental HRS was established in BDL rats. Accompanied with the decreased renal arterial blood flow and renal function, the HO-1 mRNA and protein levels in kidney of rats were significantly lower in cirrhotic group than in sham group, indicating that production of CO is decreased in kidney, because CO is mainly generated by degrading heme due to HO. HO has constitutive and inducible isoforms[25,26]. HO-1, a 32-kDa inducible protein[27], catalyzes the rate-limiting step in oxidative degradation of heme to biliverdin, releasing equimolar amounts of CO and iron[26]. The HO-1/CO system plays a vital role in many activities, including anti-oxidative stress, anti-inflammation, inhibition of cellular proliferation, and regulation of cytokine expression. CO, a gaseous messenger similar to NO, can activate soluble guanylate cyclase leading to production of cGMP[28] which mediates various physiological functions[29] including vasodilation[30]. CO can also relax vascular smooth muscle in a cGMP-independent manner[12,13]. HO-1 activity is the primary source of circulating CO[31], and HO-1 contributes to vasodilation mainly through HO-1-derived CO[32]. Thus, the declined HO-1 expression in kidney may be responsible for a decrease in vasodilation. In addition, oxidants can cause localized renal vasoconstriction[33]. Thus, the antioxidant action of HO-1 and its products can preserve renal arterial blood flow. Decreased HO-1 expression in kidney of BDL rats impairs their ability to buffer locally produced oxidants, thus leading to decreased renal arterial blood flow and deteriorated renal function.

Surprisingly, the COHb level was significantly higher in cirrhotic group than in sham group, suggesting that there is more CO in circulation, since it is predominately bound to hemoglobin in the form of COHb[34]. This large amount of CO may be produced by increased HO-1 expression in other organs, such as liver, because the HO-1 expression level in liver was higher in cirrhotic group than in sham group. Thus, we speculate that the decreased HO-1 expression in kidney and suppression of locally produced CO contribute to the decreased renal arterial blood flow and renal dysfunction in cirrhotic rats.

To evaluate the functional consequences of HO-1 changes, BDL rats were treated with either ZnPP or CoPP. The PVP and MAP were measured to evaluate systemic effects of ZnPP and CoPP treatment, renal arterial blood flow, 24 h total urinary volume, serum and urine levels of sodium and Cre were also measured to evaluate the effects of ZnPP and CoPP on HRS. The expression level of HO-1 mRNA and protein in kidney was lower in ZnPP treatment group and higher in CoPP treatment group than in cirrhotic group, without obvious changes in PVP and MAP, while the renal arterial blood flow was significantly lower and the renal function was more severely impaired in ZnPP treatment group than in cirrhotic group, as demonstrated by the decreased 24 h total urinary volume, Ccr, and serum level of sodium. In contrast, the renal arterial blood flow and 24 h total urinary volume were higher in CoPP treatment group than in cirrhotic group. However, unlike ZnPP treatment, CoPP treatment did not significantly affect serum Cre or Ccr compared with cirrhotic group. Nevertheless, increasing the treatment time or the CoPP dose may have elicited the different results in our study. Because ZnPP or CoPP treatment did not significantly affect MAP or PVP, changes in renal artery vascular tone were not considered to represent systemic vascular effects of HO-1 inhibition or HO-1 induction.

In conclusion, renal HO-1 expression, renal arterial blood flow, Ccr, 24 h total urinary volume, serum and urine sodium concentrations are lower in cirrhotic rats than in sham rats. Inhibition of renal HO-1 activity decreases renal arterial blood flow and aggravates renal dysfunction in rats with HRS. Meanwhile, activation of renal HO-1 activity had the opposite effects. Taken together, decreased HO-1 expression in kidney plays an important role in the pathogenesis of experimental HRS.

COMMENTS

Background

Renal dysfunction is very common in patients with advanced cirrhosis, and its severity ranges from electrolyte-balance disturbances and water retention to hepatorenal syndrome (HRS). HRS is a poor prognostic indicator for patients with liver cirrhosis, and can increase its morbidity and mortality in such patients. At present, the pathophysiological mechanism underlying HRS is still incompletely understood.

Research frontiers

Intense renal vasoconstriction in combination with peripheral arterial vasodilation plays an important role in occurrence of HRS. Studies have shown that the heme oxygenase-1 (HO-1)/carbon monoxide system is a crucial component in regulation of vascular tone and that inhibition of HO-1 blocks vasodilation induced by heme. HO-1 is also central to the prevention of renal failure after renal ischemia or glycerol-induced acute renal injury in rats. In this study, we in-

vestigated the expression of HO-1 in the kidney of experimental rats with HRS and evaluated the functional role of HO-1 in the pathogenesis of HRS.

Innovations and breakthroughs

The characteristics of HRS became evident in rats 4 wk after bile duct ligation (BDL), including reduced creatinine clearance rate and fluid retention, without changes in renal histology. The decreased renal arterial blood flow and renal function were accompanied with decreased renal expression of HO-1 at mRNA and protein levels. To evaluate the functional consequences of the changes in HO-1 expression, the authors treated BDL rats with either zinc protoporphyrin IX (ZnPP), a specific HO-1 inhibitor or cobalt protoporphyrin (CoPP), a HO-1 inducer. ZnPP treatment significantly reduced the renal arterial blood flow and further worsened the renal function, while CoPP treatment had the opposite effects. The relation between HO-1 and renal arterial blood flow and renal function was systematically evaluated by treating BDL rats with ZnPP and CoPP.

Applications

The mechanisms underlying renal dysfunction in patients with advanced liver disease or cirrhosis are complicated and remain incompletely understood. However, the findings in this study indicate that decreased renal HO-1 expression plays an important role in the pathogenesis of experimental HRS.

Terminology

HRS, a progressive renal failure that occurs in patients with chronic liver disease and advanced hepatic failure in the absence of any apparent clinical cause for renal insufficiency, corresponds to a functional alteration without histological changes in renal tissue. HO-1 is heme oxygenase-1, a rate-limiting enzyme that is also known as heat shock protein 32, and can be induced by CoPP and inhibited by ZnPP *in vivo*.

Peer review

This paper, written in rather good English, is quite important and interesting, which shows that decreased HO-1 expression in the kidney plays an important role in the pathogenesis of experimental HRS, as it demonstrated that the renal HO-1 expression, renal arterial blood flow, creatinine clearance rate, 24 h total urinary volume, serum and urine sodium concentrations were lower in cirrhotic rats than in sham rats, thus inhibition of renal HO-1 activity decreases renal arterial blood flow and aggravates renal dysfunction in rats with HRS.

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BRIEF ARTICLE

Low red blood cell levels of deglycating enzymes in colorectal cancer patients

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Abstract

AIM: To investigate Glyoxalase I and fructosamine-3-kinase (FN3K) activity in red blood cells from patients with colorectal adenomas and cancer.

METHODS: Thirty three consecutive subjects with one or more histologically confirmed colorectal adenomatous polyps, 16 colorectal cancer patients and a group of 11 control subjects with normal colonoscopy were included in the study. Glyoxalase I and FN3K activities were measured in red blood cells using a spectrophotometric and radiometric assay, respectively.

RESULTS: A significant reduction in both Glyoxalase I and FN3K activity was detected in patients with tumors compared to patients with adenomas and the controls. Erythrocyte Glyoxalase I activity in colorectal cancer was approximately 6 times lower than that detected in patients with adenoma (0.022 ± 0.01 mmol/min per milliliter *vs* 0.128 ± 0.19 mmol/min per milliliter of red blood cells, $P = 0.003$, Tukey's test). FN3K activity in red blood cells from patients with colon cancer was approximately 2 times lower than that detected in adenoma patients (19.55 ± 6.4 pmol/min per milliliter *vs* 38.6 ± 31.7 pmol/min per milliliter of red blood cells, $P = 0.04$, Tukey's test).

CONCLUSION: These findings suggest that deglycating enzymes may be involved in the malignant transformation of colon mucosa.

Key words: Colorectal cancer; Enzymatic activity; Fructosamine-3-kinase; Glycation; Glyoxalase I

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INTRODUCTION

The enzymatic defense against glycation involves enzymatic activities, such as the glyoxalase system, amadoriase and fructosamine 3 kinase, which suppress the formation of glycation adducts and repair sites of early glycation[1].

Glyoxalase I together with Glyoxalase II constitutes the glyoxalase system, a ubiquitous detoxification pathway which protects against cellular damage caused by potent cytotoxic metabolites, such as methylglyoxal. Methylglyoxal is a physiological substrate, derived from glycolysis, *via* degradation of triose phosphate intermediates, lipid peroxidation, and fragmentation of glycated proteins[2].

As a highly reactive metabolite, methylglyoxal has a strong ability to cross-link with protein amino groups to form stable products called advanced glycation end products, and to attack guanine residues of DNA leading to DNA glycation[3]. The cytotoxicity of methylglyoxal is due to its mutagenic and antiproliferative properties and to its ability to trigger apoptosis[4], *via* oxidative signaling[5]. Experimental evidence shows that the glyoxalase system is involved in the regulation of cellular growth[6,7]. Altered expression of this system is involved in several human disorders, including cancer[8-11]. Over-expression of Glyoxalase I is associated with clinical multidrug resistance in tumors of high incidence and mortality, such as carcinomas of the lung, breast and prostate and Glyoxalase I inhibitors provide effective therapy for these tumors[1,12].

Fructosamine-3-kinase (FN3K) is an intracellular deglycating enzyme that phosphorylates fructosamines on the third carbon of their deoxyfructose moiety. The fructosamine 3-phosphates so formed are unstable and their spontaneous decomposition leads to the regeneration of the free amine[13,14]. This enzyme seems to catalyze a repair mechanism offering selective cell advantage.

We previously evaluated *FN3K* gene expression in colorectal cancer patients, and showed that *FN3K* gene expression was significantly lower in colon cancer tissue than in the corresponding surrounding normal mucosa[15]. Moreover, we found that FN3K activity is particularly downregulated in tumors located on the left side of the colon[16].

The adenoma-carcinoma sequence in the colon represents one of the most characterized models of human tumor progression. The transition from normal to malignant phenotype implies the activation of pathways that underlie aberrant clone expansion[17].

Alterations of metabolic pathways, such as changes in the balance between glycation and enzymatic anti-glycation defense, are considered to be crucial for sustaining tumor development[18]. The risk of colorectal adenoma increases with serum levels of fructosamine[19]. The decline in expression of deglycating enzymes may be the key to increased protein glycation in the tumor phenotype.

In this study, we evaluated the levels of Glyoxalase I and FN3K activity in red blood cells from patients with colorectal adenomas and cancer.

MATERIALS AND METHODS

Subjects

The study included thirty three consecutive subjects (18 males and 15 females, mean age 67.6 ± 11.7 years) with one or more histologically confirmed colorectal adenomatous polyps removed after complete endoscopy, and sixteen colorectal cancer patients (6 males and 10 females, mean age 68.1 ± 6.4 years) undergoing colon surgery. A group of eleven control subjects (6 males and 5 females, mean age 45 ± 5.8 years) with normal colonoscopy, performed in the same endoscopy unit during the same period, was also included.

Written informed consent was obtained from all the participants.

Measurement

Anthropometric measurements were obtained by the participants wearing scrub suits without shoes. Body weight was measured using a calibrated scale (Detecto; model 437). Standing height was measured with a vertical metal ruler. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m^2).

Ficoll-Paque separation

Participants were fasted for 12 h prior to examination. Blood samples taken from the subjects by venous puncture were collected in tubes containing EthyleneDiamineTetraacetic Acid (K-EDTA) anticoagulant or a serum separator gel. Blood serum was shipped to the central laboratory for routine analyses. For *in vitro* isolation of erythrocytes, blood samples with K-EDTA were quickly layered on the Ficoll-Paque solution and centrifuged at 400 *g* for 40 min at 20℃. The lymphocytes and plasma were then removed and the erythrocytes were recovered from the bottom layer and washed with 4-volumes of phosphate-buffered saline. Isolated red blood cells were stored at -80℃ until assayed. All the analyses were performed within 6 mo.

Glyoxalase *I* activity assay

Glyoxalase I enzymatic activity was measured in frozen erythrocytes, according to the method described by Thornalley[20] with minor modifications. The frozen red blood cell pellet was lysed with 1 mL of 10 mmol/L TRIS-HCl, pH = 7.8, 1 mmol/L DTT, 1 μg/mL leupeptin and was well mixed. The samples were centrifuged for 10 min at 2000 *g* and the supernatant was used for the enzymatic activity assay. Aliquots of 50 μL of supernatant were incubated with 100 μL of reaction mix containing 7.9 mmol/L methylglyoxal, 1 mmol/L glutathione, 14.6 mmol/L magnesium sulfate and 182 mmol/L imidazole HCl, pH = 7.0. The activity of Glyoxalase I was determined by monitoring the increase in absorbance at 240 nm due to the formation of *S*-D-lactoylglutathione for 2 min at 25℃. One unit of activity was defined as the formation of 1 mmol of *S*-D-lactoylglutathione/min per milliliter of blood red cells.

Fructosamine-3 kinase activity assay

FN3K enzymatic activity was measured in frozen erythrocytes lysed with 1 mL of 10 mmol/L TRIS-HCl, pH = 7.8, 1 mmol/L DTT and 1 μg/mL leupeptin. The samples were centrifuged for 10 min at 2000 *g* and 50 μL of supernatant were incubated with 100 μL of reaction mix [5 mmol/L glucose, 10 mmol/L Tris-HCl (pH 7.8), 1 mmol/L DTT, 1 μg/mL leupeptin, and 2 mmol/L D-[1-^{14}C]-glucose (49.5 mCi/mmol) for 40 min at 37℃.

Subsequently, 30 μL aliquots of the samples were spotted on cation-exchange papers (P81; Whatman), which were washed three times with ice-cold 75 mmol/L H_3PO_4 and then once with alcohol and once with acetone. After drying, the papers were counted for radioactivity in the presence of a scintillant. FN3K activity was expressed as picomoles of incorporated D-[1-^{14}C]-glucose/min per milliliter of red blood cells. Parallel samples were as-

sayed to evaluate total and non-specific radioactivity. The enzyme activity assay was validated using samples in the presence of the FN3K inhibitor, 1-deoxy-1-morpholino-fructose.

Statistical analysis

The mean and standard deviation were calculated for each group. Groups were compared using one-way analysis of variance and Tukey's Multiple Comparison test. Differences in the means were considered statistically significant if the *P*-value was < 0.05. Analysis of covariance was used to model, controlling for glycemia, a potential variable confounder.

RESULTS

The clinical characteristics of all subjects studied are shown in Table 1. There was a weak increase in glycemia levels from the controls to the adenoma and cancer patients. No difference in mean BMI values between the groups was observed.

Table 2 summarizes the data for Glyoxalase I and FN3K activity levels detected in red blood cells from controls, adenoma and tumor-bearing patients.

There was a significant reduction in Glyoxalase I activity in red blood cells from patients with tumor compared to controls, and a trend in decreasing activity from controls to adenoma and cancer patients. Erythrocyte Glyoxalase I activity in colorectal cancer was about 6 times lower than that detected in patients with adenoma (0.022 ± 0.01 mmol/min per milliliter *vs* 0.128 ± 0.19 mmol/min per milliliter of red blood cells, $P = 0.003$, Tukey's test). A significant reduction in FN3K activity levels in erythrocytes from colon cancer patients with respect to controls and adenoma patients was also observed. FN3K activity in the red blood cells of patients with colon cancer was approximately 2 times lower than that detected in adenoma patients (19.55 ± 6.4 pmol/min per milliliter *vs* 38.6 ± 31.7 pmol/min per milliliter of red blood cells, $P = 0.04$, Tukey's test).

The differences in enzymatic activities among the control, adenoma and cancer groups were controlled for fasting glycemia using analysis of covariance. The absolute value of association did not decrease; there was only an increase in standard error of the coefficients and consequently a decrease in the *P*-value of the null hypothesis. In any case, the inverse association of both enzymes with cancer was still statistically significant (two tails, $P < 0.05$).

Table 1 Clinical characteristics of subjects enrolled in the study (mean ± SD)

	Control subjects	Adenoma patients	Cancer patients
n	11	33	16
Age (yr)	45 ± 5.8	67.6 ± 11.7	68.1 ± 6.4
Female/male	5/6	15/18	10/6
Glycemia (mmol/L)	4.87 ± 0.2	5.82 ± 1.2	7.06 ± 2.4
BMI (kg/m^2)	25.6 ± 7.7	27.8 ± 6.3	27.1 ± 3.1

BMI: Body mass index.

Table 2 Red blood cell levels of Glyoxalase I and fructosamine-3-kinase activity in controls, adenoma and tumor-bearing patients (mean ± SD)

	Glyoxalase I	FN3K
Controls (*n* = 11)	0.173 ± 0.25	29.05 ± 14.6
Adenomas (*n* = 33)	0.128 ± 0.19[a]	38.6 ± 31.7[b]
Tumors (*n* = 16)	0.022 ± 0.01[a]	19.55 ± 6.4[b]

[a,b]$P < 0.05$, Tukey's test. The glyoxalase enzymatic activity is expressed as mmol/min per milliliter of red blood cells and the enzymatic activity of FN3K is expressed as pmol/min per milliliter of red blood cells. FN3K: Fructosamine-3-kinase.

DISCUSSION

This study provides evidence of a role for the deglycating enzymes, Glyoxalase I and FN3K, in colorectal cancer development. The trend of decreased Glyoxalase I activity from the controls to adenoma and cancer patients strengthens the association between this deglycating enzyme and colon cancer, and suggests that its decrease in activity can support the evolution of the malignant process.

The glyoxalase system has received considerable attention regarding its possible relationship with cancer. In an animal model of carcinogenesis, an appreciable decrease in rat liver glyoxalase activity was found after the development of hepatoma[21]. Some authors also showed that glyoxalase activity in the blood of tumor-bearing animals was much lower than glyoxalase activity in the blood of normal animals[22]. The presence of Glyoxalase I gene polymorphism, which may result in a decrease in glyoxalase activity, increases breast cancer risk[23]. This gene polymorphism seems to have a role not only in the development of breast cancer, but also in the progression of neoplasia[23].

Recently, a significant reduction in *FN3K* gene expression was detected in colorectal cancer with respect to normal pair-matched tissue[15,16], suggesting that decreased FN3K expression is related to the malignant phenotype.

This study also suggests that there are functional modifications of FN3K in colon cancer development, since reduced FN3K activity is detectable in the progression from adenoma to cancer.

Epidemiological studies clearly indicate that the risk of several types of cancer (including pancreas, liver, breast, colorectal, and urinary tract) is increased in diabetic patients[24,25]. Higher levels of serum glucose were present in our patients with colon cancer compared to those with adenoma and the controls. The inverse association of both enzymes with cancer was maintained after controlling for fasting glycemia. However, we doubt the necessity to control for glycemia, because it may be part of the causal chain between the enzymes and the neoplasia, as an intermediate variable. Hyperglycemia may correlate with the development of adenoma and invasive colon cancer[26], and non-enzymatic glycation is one of the principal

mechanisms by which hyperglycemia contributes to cellular damage[27].

The enzymatic defense against glycation suppresses damage to biological macromolecules, if this defense in normal physiological states is at a low level, cellular injury occurs. Glycation proteins with associated functional impairment of repair enzymes has a critical role in the activation of pathways of cellular transformation[18,28].

The findings of this study provide evidence that deglycating enzymes may be involved in increasing the risk of precancerous lesions and malignant transformation of colon mucosa.

Further studies on a large cohort of patients with colorectal cancer will be designed to translate our findings into clinical practice, allowing the development of a fast and accurate blood test to diagnose colorectal cancer at different stages.

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COMMENTS

Background

Protein glycation is a spontaneous reaction involving reducing sugars with the amino groups of proteins. In this reaction the ε - amino group of lysine condenses with the carbonyl of a sugar to form a Schiff's base, which then slowly undergoes an Amadori rearrangement to become a ketoamine or, if the reacting sugar is glucose, fructosamines. The fate of fructosamines is to continue to react slowly until they become "advanced glycation end products" which are thought to play a role in the pathophysiology of several human disorders, including cancer. Protein glycation contributes to the morbidity and mortality of diseases which have a major social impact (diabetes, heart disease and endstage renal disease) and is suspected to contribute to other diseases (Alzheimer's disease, arthritis and ageing). It is now recognized that there is an enzymatic defense against glycation - a group of enzymes which suppress the physiological levels of potent glycating agents and repair glycated proteins, such as Glyoxalase I and fructosamine-3-kinase.

Research frontiers

Alterations of metabolic pathways, such as changes in the balance between glycation and enzymatic anti-glycation defense, are considered to be crucial in the activation of pathways of cellular transformation. This study identifies molecular targets which can be used not only for colorectal cancer diagnosis, but also for its prevention and treatment.

Innovations and breakthroughs

The study of circulating levels of deglycating enzymes in red blood cells from patients with colorectal cancer is certainly an innovation that might help to diagnose colorectal cancer using a fast and partially invasive method.

Applications

Further studies on a large cohort of patients with colorectal cancer will be designed to translate our findings into clinical practice, allowing the development of a fast and accurate blood test to diagnose colorectal cancer at different stages.

Peer review

This is a nicely presented concise research study on the assessment of Glyoxalase I and fructosamine-3-kinase activity in red blood cells from colorectal cancer patients. The experimentation is well performed. A clinical relevant experimental group of cancer and healthy patients are investigated in this study. Statistical methods are careful chosen and resulted in significant clinical relevant observations. The study of deglycating enzymes in red blood cells may be translate in clinical practice by the development of a fast accurate test to diagnose colorectal cancer in different stages.

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BRIEF ARTICLE

Bones and Crohn's: No benefit of adding sodium fluoride or ibandronate to calcium and vitamin D

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Abstract

AIM: To compare the effect of calcium and cholecalciferol alone and along with additional sodium fluoride or ibandronate on bone mineral density (BMD) and fractures in patients with Crohn's disease (CD).

METHODS: Patients (n =148) with reduced BMD (T-score < -1) were randomized to receive cholecalciferol (1000 IU) and calcium citrate (800 mg) daily alone(group A, n = 32) or along with additional sodium fluoride (25 mg *bid*) (group B, n = 62) or additional ibandronate (1 mg iv/3-monthly) (group C, n = 54). Dual energy X-ray absorptiometry of the lumbar spine (L1-L4) and proximal right femur and X-rays of the spine were performed at baseline and after 1.0, 2.25 and 3.5 years. Fracture-assessment included visual reading of X-rays and quantitative morphometry of vertebral bodies (T4-L4).

RESULTS: One hundred and twenty three (83.1%) patients completed the first year for intention-to-treat (ITT) analysis. Ninety two (62.2%) patients completed the second year and 71 (47.8%) the third year available for per-protocol (PP) analysis. With a significant increase in T-score of the lumbar spine by +0.28 ± 0.35 [95% confidence interval (CI): 0.162-0.460, P < 0.01], +0.33 ± 0.49 (95% CI: 0.109-0.558, P < 0.01), +0.43 ± 0.47 (95% CI: 0.147-0.708, P < 0.01) in group A, +0.22 ± 0.33 (95% CI: 0.125-0.321, P < 0.01); +0.47 ± 0.60 (95% CI: 0.262-0.676, P < 0.01), +0.51 ± 0.44 (95% CI: 0.338-0.682, P < 0.01) in group B and +0.22 ± 0.38 (95% CI: 0.111-0.329, P < 0.01), +0.36 ± 0.53 (95% CI: 0.147-0.578, P < 0.01), +0.41 ± 0.48 (95% CI: 0.238-0.576, P < 0.01) in group C, respectively, during the 1.0, 2.25 and 3.5 year periods (PP analysis), no treatment regimen was superior in any in- or between-group analyses. In the ITT analysis, similar results in all in- and between-group analyses with a significant in-group but non-significant between-group increase in T-score of the lumbar spine by 0.38 ± 0.46 (group A, P < 0.01), 0.37 ± 0.50 (group B, P < 0.01) and 0.35 ± 0.49 (group C, P < 0.01) was observed. Follow-up in ITT analysis was still 2.65 years. One vertebral fracture in the sodium fluoride group was detected. Study medication was safe and well tolerated.

CONCLUSION: Additional sodium fluoride or ibandronate had no benefit over calcium and cholecalciferol alone in managing reduced BMD in CD.

Key words: Crohn's disease; Bone mineral density; Vertebral fracture; Cholecalciferol; Calcium; Ibandronate; Sodium fluoride

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INTRODUCTION

Inflammatory bowel disease (IBD) patients are at risk of reduced bone mineral density (BMD), especially in Crohn's disease (CD)[1-5]. Genetic, endocrine, metabolic and nutritional factors contribute to CD-associated osteoporosis, and inflammation *per se* may exert an important risk since inflammatory mediators such as the pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1β or IL-6 and other TNF-related cytokines such as receptor activator of nuclear factor κB (RANK) and its ligand, RANKL or osteoprotegerin, are directly involved in the disease process[6-13].

The prevalence of a reduced BMD in IBD patients is up to 38% with some 15% suffering from osteoporosis[1-5,7]. Thus, of approximately 300 000 patients with IBD in Germany[14], up to 45 000 may have an increased fracture risk. The high prevalence of up to 21.7% in osteoporosis-related vertebral fractures is of clinical relevance[1,2,15-18].

Different strategies to improve BMD and to prevent osteoporosis-related fractures have been examined. Hormone replacement therapy (HRT) and bisphosphonates are established in postmenopausal osteoporosis and bisphosphonates in steroid-induced osteoporosis[19-21]. In particular, the efficacy of bisphosphonates has received special interest in large clinical trials[22-24]. Bisphosphonates have reduced the fracture risk considerably in patients with postmenopausal osteoporosis[25,26]. Sodium fluoride can also increase BMD but its efficacy in reducing fractures remains controversial[27-29].

To this day, few studies have evaluated the management of reduced BMD in IBD patients. Calcium and vitamin D administration can inhibit the rate of bone loss[30]. HRT is an effective treatment to prevent bone loss in postmenopausal women with CD[31]. In a previous study, we demonstrated the efficacy of sodium fluoride in increasing BMD in CD patients[32]. Other studies reported a significant increase in BMD with the administration of iv pamidronate (30 mg every 3 mo)[33], alendronate (10 mg/d)[34] or etidronate periodically (400 mg orally for 14 d)[35]. However, the primary end-point in all studies was BMD and only small cohorts with limited follow-up were investigated; the prevalence and incidence of vertebral fractures was not evaluated.

Our aim was to assess the effectiveness of cholecalciferol and calcium alone or with additional sodium fluoride or ibandronate in a larger CD patient population and longer follow-up period. The primary endpoint was to assess the efficacy of the 3 therapeutic approaches to improve BMD (in-group change). Secondary endpoints were to compare the 3 therapies for the best improvement in BMD (between-group change), fracture rate and safety.

MATERIALS AND METHODS

Patients

The 148 randomized outpatients had a diagnosis of CD based on histological, endoscopic, radiological or clinical criteria and a reduced BMD of the lumbar spine: T-score < -1, i.e. osteopenia according to World Health Organization (WHO) criteria as published in 1994[36]. Disease-related data on previous and current state of health were recorded using a standardized questionnaire throughout the study including adverse effects and serious adverse effects reporting. Disease activity was estimated using the CD activity index (CDAI)[37]. Cumulative lifetime steroid-dose was estimated and expressed in grams of prednisolone equivalent. Nutritional status was assessed by body mass index (BMI). Exclusion criteria included: age < 18 years, chronic renal insufficiency (creatinine > 1.5 mg/dL), known primary hypo- or hyperparathyroidism, untreated thyroid disease, and any known medication, e.g. previous treatment with either sodium fluoride or bisphosphonates, or a condition affecting BMD other than glucocorticoid therapy. None of the patients was pregnant and female patients planning pregnancy were excluded.

Ethics

The study was approved by the Ethics Committee of the University of Ulm/Germany, and conducted in accordance with the 1975 Helsinki Declaration, as revised in 1983. All participants gave written informed consent before inclusion.

Protocol, assignment and masking

Patients were randomized to treatment group A, B or C, taking study medication as follows: (1) 1000 IU cholecalciferol (Vigantoletten®, Merck, Darmstadt, Germany) and 800 mg calcium citrate (Calcitrat®, Merckle, Ulm, Germany) daily (group A); (2) additional 25 mg of slow-release sodium fluoride (Nafril®, Merckle, Ulm, Germany) *bid* (group B); and (3) additional ibandronate 1 mg iv 3-monthly (Bondronat®, Roche, Basle, Switzerland) (group C). A random 1:2 allocation sequence, basic cholecalciferol and calcium (A) and additional sodium fluoride or additional ibandronate (B or C), was computer-generated and the sequences were concealed until intervention was assigned. Baseline examination included dual energy X-ray absorptiometry (DXA) of the spine and femur and plain radiographic imaging of the thoracic and lumbar spine in 2 planes. Follow-up examinations were conducted at 3-mo intervals. In group B, sodium fluoride was taken daily for 12 mo, followed by a 3-mo fluoride-free period. The second and third 12-mo cycle started at month 15 and 30. Follow-up DXA and plain radiography of the spine were performed after 12, 27 and 42 mo, i.e. 1.0, 2.25 and 3.5 years. With the last patient in study in June 2005, this patient completed the 3.5-year study period in January 2009 (last patient out).

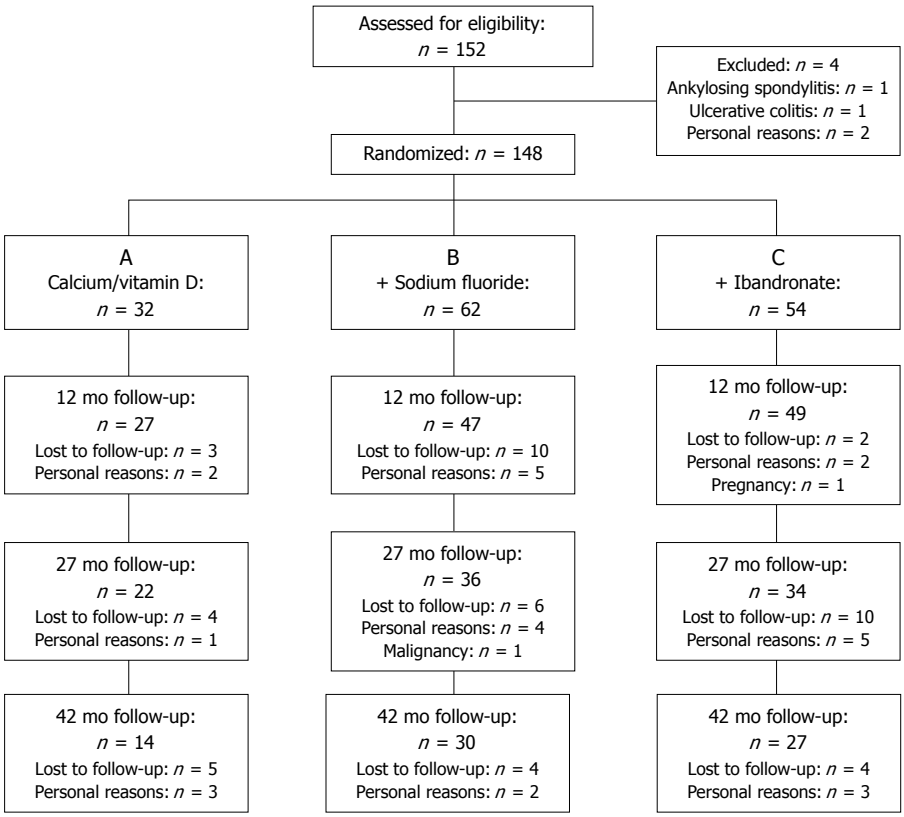

Figure 1 CONSORT diagram.

Bone densitometry

BMD of the spine (L1-L4) was assessed by DXA (Hologic QDR1000, Hologic Inc., Waltham/MA). At the proximal right femur, 4 sites (femoral neck, trochanter and intertrochanteric area, Ward's triangle) were measured; an average (total femur) was obtained from the first 3 sites. Average BMD values for L1-L4 and total femur were used for calculations. The manuacturer supplied the normal values. BMD was expressed as absolute values (g/cm^2) and as number of standard deviations from the peak bone mass of a young adult gender-matched reference population (T-score). According to the WHO recommendation for postmenopausal women as published in 1994, reduced BMD was defined as a T-score < -1.0[36]. Patients with e.g. major sclerosis of the aorta, osteophytes and scoliosis on X-rays precluding accurate measurements of lumbar BMD by DXA were excluded.

Quantitative morphometry

Morphometric methods have been developed for standardized assessment of vertebral deformities in studies of spinal osteoporosis[38]. The use of a fixed percentage reduction in vertebral height is the simplest and most practical method to study vertebral deformities[39]. In this study, visual reading of X-rays and the quantitative morphometry (QM) of the vertebral bodies were standardized according to criteria of the European Vertebral Osteoporosis Study[40]; only the threshold value was set from 25% to 20%. QM was performed using 6-point digitization to calculate the anterior (Ha), mid (Hm), and posterior (Hp) height of the vertebral bodies T4-L4 (Figure 1). A vertebra was classified deformed if at least one ratio (Ha/Hp, Hm/Hp, Hp/Hp-up and Hp/Hp-low) was below the threshold value. For every vertebra considered deformed quantitatively, a radiological differential diagnosis was performed for the etiology, distinguishing osteoporotic, degenerative, traumatic and other reasons. Differential diagnosis prevents overestimation of prevalent osteoporotic fractures due to deformations of other etiology, since 45.9% and 30.9% of spinal deformities in men and women are reported to be of non-osteoporotic origin[41].

Laboratory testing

A patient's hematocrit was determined for the calculation of the CDAI, and other inflammation-related parameters [leukocytes, platelets, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)] were obtained. Regarding bone metabolism, we focused on calcium phosphate homeostasis and investigated calcium and phosphate as well as the 25(OH)- and 1,25(OH)$_2$-vitamin-D$_3$ serum levels and parathyroid hormone. All laboratory tests were performed in the DIN EN ISO 15189:2007 accredited "Zentrale Einrichtung Klinische Chemie" of the University Hospital of Ulm, Germany. Laboratory technology and standard values can be checked at http://www.uniklinik-ulm.de/index.php?id=1159.

Statistical analysis

Results are presented as mean ± SD. Qualitative variables were expressed as frequencies and percentage. The Mann-

Whitney rank sum test was used to test the effect of each therapy on BMD and biochemical markers after 12, 27 and 42 mo compared to baseline. The Student *t*-test for unpaired observations was used to compare between-group differences. Intention-to-treat (ITT) analysis was performed for all patients with at least one DXA during follow-up. Two-tailed tests for significance were used in the statistical analyses and $P \leqslant 0.05$ was considered significant. The Statistical Package SAS V6.11 was used for analysis.

RESULTS

Participant flow and follow-up

The CONSORT diagram shows the number of patients randomly assigned and receiving intended treatment, the patient flow through each year of the study, the number completing the study protocol, and the number analyzed for the primary outcome (Figure 1). One hundred and forty-eight patients with a T-score < -1.0 were ITT analysis, 92 (62.2%) completed the 27-mo and 71 (47.8%) the 42-mo study period and were available for per-protocol (PP) analysis. Reasons for withdrawal were failure to attend follow-up [48 patients (32.4%)] and personal reasons [27 patients (18.2%), withdrawal of written informed consent ($n = 7$), referred to primary care ($n = 10$), moving house ($n = 7$), unknown ($n = 3$)]. One patient was excluded due to a malignancy (testicular cancer), retrospectively present before randomization; he recovered completely.

Baseline characteristics

Baseline characteristics of the patients are given in Table 1. With a 1:2 random allocation to treatment groups, group A was smaller compared to group B or C. BMD was slightly but non significantly higher in group A. Patients in group A were a little younger than in group B ($P = 0.3$) and C ($P = 0.06$). No further differences in baseline characteristics were observed.

BMD of the spine, in-group change

In group A, BMD of the spine increased continually during the 1.0, 2.25 and 3.5-year study period (Table 2, Figure 2). In group B, lumbar BMD increased during the 1.0 and 2.25-year period, and in the third year, a further but non significant increase was observed (Table 2, Figure 2). In group C, BMD of the spine increased continually during the 1.0, 2.25 and 3.5-year period, again with the greatest increases in the first and second year (Table 2, Figure 2).

BMD of the spine, compared between-groups

Comparing the increase in lumbar spine BMD of the groups A, B and C at 1.0, 2.25 and 3.5 years, no group revealed superior results. There was no difference for group B receiving added sodium fluoride or for group C receiving added ibandronate in comparison with group A receiving only cholecalciferol and calcium citrate nor was there a significant difference in the comparison of groups B and C at any time in the 3.5-year study period (Table 2, Figure 2).

Table 1 Baseline characteristics (mean ± SD) *n* (%)

	Group A calcium/ vitamin D	Group $B_{0/1}$ + sodium fluoride	Group $C_{0/1}$ + ibandronate
No. of patients	32	62	54
Male/female	14/18	29/33	27/27
Age (yr)	33.8 ± 9.76	35.7 ± 12.8	36.8 ± 13.1
Duration of disease (yr)	7.4 ± 1.7	9.4 ± 2.1	8.1 ± 1.9
Smoking	13 (40.6)	23 (37.1)	19 (35.2)
Postmenopausal	0	2	1
Extent of disease			
Ileal disease	11 (34.4)	20 (32.3)	21 (38.9)
Colonic disease	5 (15.6)	8 (12.9)	7 (13)
Ileocolonic disease	16 (50.0)	34 (54.8)	26 (48.1)
Bowel resection			
No bowel resection	18 (56.2)	39 (62.9)	29 (53.7)
Ileal resection	8 (25.0)	12 (19.4)	14 (25.9)
Colonic resection	2 (6.2)	5 (8.1)	4 (7.4)
Ileocolonic resection	4 (12.6)	6 (9.7)	7 (13)
Patients with bowel resection during study	4	6	5
Use of corticosteroids			
No previous use	3 (9.4)	6 (9.7)	6 (11.1)
Cumulative dose < 10 g	22 (68.8)	36 (58.1)	34 (63)
Cumulative dose > 10 g	7 (21.8)	28 (32.2)	14 (25.9)
Body weight (kg)	69.4 ± 15.51	63.71 ± 11.9	64.8 ± 13.91
Body height (cm)	172 ± 7.63	170 ± 8.8	170 ± 9.0
BMI (kg/m^2)	23.54 ± 5.34	22.01 ± 3.6	22. 4 ± 3.92
CDAI	141.5 ± 100.96	145 ± 95.5	135.9 ± 85.03
T-score spine	-1.57 ± 0.31	-1.82 ± 0.75	-1.89 ± 0.71
BMD spine (g/cm^2)	0.90 ± 0.04	0.87 ± 0.09	0.85 ± 0.08
Pre-existing vertebral fractures[1]	6 (22.2) of patients with 10 fractures	9 (19.2) of patients with 18 fractures	14 (28.6) of patients with 28 fractures

[1]Intention to treat (ITT) analysis. BMI: Body mass index; CDAI: Crohn's disease activity index; BMD: Bone mineral density.

BMD of the femur, in-group change and compared between-groups

There was no significant change in femur BMD in any of the 3 groups during the entire follow-up period, and no significant differences between groups A, B and C at 1.0, 2.25 and 3.5-year follow-up in the change in femur BMD (data not shown).

BMD of the spine (ITT)

A pre-planned ITT analysis was performed. As in PP analysis, comparing the increase in BMD in in- and between-group A, B and C analysis, cholecalciferol and calcium alone did not perform any worse than with additional sodium fluoride or ibandronate, and no group revealed superior results (Table 3, Figure 3) Mean observation time in the ITT analysis was 2.65 years.

Prevalence and incidence of vertebral fractures

For assessment of prevalent fractures and fracture incidence, the ITT population was analyzed, i.e. 123 (83.1%) patients who completed at least the first 12-mo follow-up. The duration of follow-up did not differ significantly for the treatment groups A, B and C. At baseline, a total of 56 vertebral fractures was seen in 29 (23.6%) of 123 patients,

Table 2 Bone mineral density of spine and femur, in- and between-group change

Lumbar spine		Baseline	First year	Second year	Third year
Group A	*n* (%)	32	27 (84.4)	22 (68.6)	14 (43.8)
calcium/vitamin D	T-score	-1.57 ± 0.31	-1.32 ± 0.42[b]	-1.21 ± 0.49[b]	-1.18 ± 0.36[b]
	Δ T-score (95% CI)		+0.28 ± 0.35 (0.162–0.460)	+0.33 ± 0.49 (0.109-0.558)	+0.43 ± 0.47 (0.147–0.708)
	BMD	0.90 ± 0.04	0.92 ± 0.05	0.94 ± 0.05	0.94 ± 0.04
Group B	*n* (%)	62	47 (75.8)	36 (58.0)	30 (48.4)
+ sodium fluoride	T-score	-1.82 ± 0.75[h]	-1.60 ± 0.84[d]	-1.40 ± 1.01[d]	-1.37 ± 0.95[d]
	Δ T-score (95% CI)		+0.22 ± 0.33 (0.125–0.321)	+0.47 ± 0.60 (0.262–0.676)	+0.51 ± 0.44 (0.338–0.682)
	BMD	0.87 ± 0.08	0.88 ± 0.09	0.90 ± 0.11	0.91 ± 0.11
Group C	*n* (%)	54	49 (90.1)	34 (63.0)	27 (50)
+ ibandronate	T-score	-1.89 ± 0.71	-1.69 ± 0.78[e]	-1.61 ± 0.83[e]	-1.56 ± 0.78[f]
	Δ T-score (95% CI)		+0.22 ± 0.38 (0.111– 0.329)	+0.36 ± 0.53 (0.147–0.578)	+0.41 ± 0.48 (0.238–0.576)
	BMD	0.85 ± 0.08	0.87 ± 0.08	0.89 ± 0.08	0.90 ± 0.08
Total	*n* (%)	148	123 (83.1)	92 (62.2)	71 (47.8)

In-treatment group change, baseline to first, second and third year: Group A, [b]$P < 0.01$; group B, [d]$P < 0.01$; group C, [e]$P < 0.025$, [f]$P < 0.01$; Between-treatment groups: [h]$P < 0.01$, group A *vs* group C. BMD: Bone mineral density.

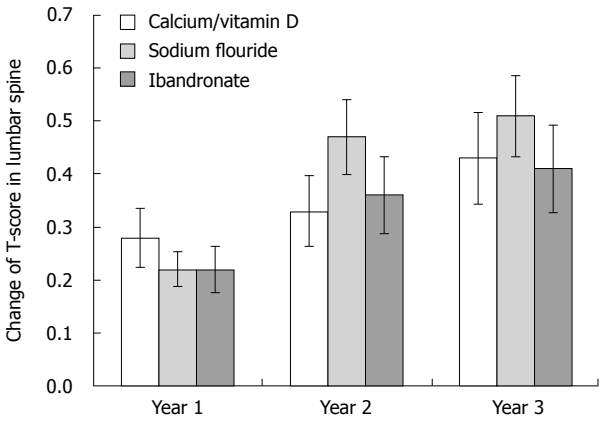

Figure 2 Change in T-score of the lumbar spine from baseline to first, second and third study year, during the 3.5-year long-term study in treatment groups A, B or C, in the per protocol population.

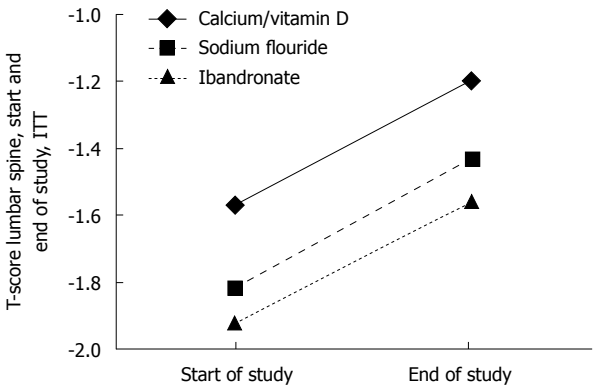

Figure 3 T-score of the lumbar spine from baseline to end of study depending in treatment groups A, B or C, in the intention to treat population. ITT: Intention-to-treat.

and one incident vertebral fracture in group B receiving sodium fluoride, but no other fractures, e.g. fractures of the hip or radius, was observed during the entire follow-up (Table 4).

Table 3 Bone mineral density of the lumbar spine, in- and between-group change (intention-to-treat)

Lumbar spine	Baseline	End of study	Δ
Group A (*n* = 27)			
T-score	-1.57 ± 0.31	-1.20 ± 0.46[b]	+0.38 ± 0.46
BMD	0.9 ± 0.04	0.94 ± 0.06	+0.04 ± 0.05
Follow-up (yr)		2.58 ± 1.0	
Group B (*n* = 47)			
T-score	-1.82 ± 0.40	-1.43 ± 0.62[b]	+0.37 ± 0.50
BMD	0.87 ± 0.05	0.91 ± 0.06	+0.04 ± 0.05
Follow-up (yr)		2.92 ± 0.89	
Group C (*n* = 49)			
T-score	-1.91 ± 0.40	-1.56 ± 0.56[b]	+0.35 ± 0.49
BMD	0.86 ± 0.04	0.90 ± 0.66	+0.04 ± 0.05
Follow-up (yr)		2.44 ± 1.17	

[b]$P < 0.01$. BMD: Bone mineral density.

Clinical course of the underlying CD and change in BMD

Seventy (57%) of the 123 patients who completed at least the first 12-mo study period were treated with systemic glucocorticoids at least once during the study, as reported in the standardized questionnaire completed at every follow-up examination at 3-mo intervals. Change in spine and femur BMD did not differ from the change observed in patients who had not received any systemic steroids (data not shown). While a slight increase in BMI and an improvement in CDAI was observed during the study period with no significant differences in and between the 3 treatment groups, the increase in BMI and the decrease in CDAI again did not correlate with the change in spine and femur BMD in all patients (data not shown).

Laboratory markers and change in BMD

No significant difference in inflammation parameters (leukocytes, platelets, ESR, CRP) were obtained in and between the groups A, B and C. Focusing on calcium-phosphate-homeostasis we investigated calcium and phosphate as well as the 25(OH)- and 1,25(OH)$_2$-vitamin-D$_3$ serum levels

Table 4 Prevalence and incidence of vertebral fractures

	All patients	Group A calcium/vitamin D	Group B + sodium fluoride	Group C + ibandronate
No. of patients	123	27	47	49
Patients with fractures, *n* (%)	29 (23.6)	6 (22.2)	9 (19.2)	14 (28.6)
No. of fractures	56	10	18	28
New fractures (*n*)	1	0	1	0
T-score lumbar spine	-1.80 ± 0.34	-1.57 ± 0.31	-1.82 ± 0.40	-1.91 ± 0.04
BMD lumbar spine (g/cm^2)	0.87 ± 0.05	0.90 ± 0.04	0.87 ± 0.05	-0.86 ± 0.04
Follow-up (yr)	2.65 ± 1.00	2.58 ± 1.00	2.92 ± 0.89	2.44 ± 1.17

BMD: Bone mineral density.

and parathyroid hormone. Only 25(OH)-vitamin-D$_3$ serum levels increased significantly in all 3 groups over time, but no change was seen in the other calcium phosphate homeostasis parameters investigated. No correlation of any serum levels of any parameter of calcium phosphate homeostasis with BMD or change in the BMD of the spine or femur could be observed (data not shown).

Adverse events

Adverse events (AEs) were reported in the standardized questionnaire used throughout the study at every 3-mo follow-up examination. AEs occurred in 35 patients (9 in group A; 14 in group B; 12 in group C). Most AEs were related to worsening of CD (28 patients), with 15 patients who had a bowel resection during study follow-up. One patient in the ibandronate group had to be withdrawn due to pregnancy (Figure 1). Study medication was generally well tolerated. Seven patients reported undigested calcium citrate and 2 undigested sodium fluoride pills in their feces. Six patients reported minor and completely reversible bone pain (< 2 h) or flu-like symptoms after intravenous infusion of ibandronate, manageable with acetaminophen if needed.

DISCUSSION

This is one of the most extended studies in the management of reduced BMD in CD. In our randomized study, we compared the effectiveness of cholecalciferol and calcium supplementation alone or along with additional sodium fluoride or additional ibandronate. More than 140 CD patients with reduced BMD (T-score < -1) were included in this study with a maximum follow-up of 3.5 years. In this young CD patient setting, increases in BMD were similar in all in- and between-treatment-group analyses, calcium and cholecalciferol supplementation not only prevented further bone loss but increased lumbar BMD and the effect was not increased further by addition of sodium fluoride or ibandronate. Regarding the prevention of fractures, the overall fracture rate in this study was too small to demonstrate between-group differences.

There were a number of limitations with the design of our study that could affect the interpretation of results. First, the study was not placebo-controlled nor blinded, and the dropout rate was high particularly after the first year. For ethical reasons we decided not to deny a basic therapeutic regimen with cholecalciferol and calcium to any patient with reduced BMD. Unfortunately, it is a flaw of the study design that there was therefore no placebo or simple observation arm. Also, by using a blinded study comparing an oral *vs* iv administrated study drug, a single tertiary outpatient clinic such as ours doing an investigator initiated trial as large as this would just be overworked. The dropout rate after the first year and only about 50% of patients completing the study reflects again the setting of our tertiary outpatient clinic where patients usually only show up if a primary or secondary health care center refer them for special reasons and problems. To manage this and to avoid misleading results we did the pre-planned ITT analysis, and found no difference in the results compared to PP analysis in- and between-group analyses and with a mean observation time of 2.65 years, which was still longer than any follow-up in the CD patient setting before.

Oral treatment of osteoporosis with bisphosphonates relies on compliance and the absorption is low, probably especially in CD patients. When we planned this study, ibandronate was the only bisphosphonate to be administered safely as an iv bolus injection, and therefore offered an interesting alternative suitable for outpatient treatment[42]. At that time, data of a study investigating 3-monthly iv injections of ibandronate in the treatment of postmenopausal osteoporosis were published, and treatment was reported to be safe and effective with a dose of 1 mg[43]. This is why we had a 1 mg ibandronate 3-monthly iv intervention arm in our study. A recent meta-analysis pooled data from 4 phase Ⅲ clinical trials to assess the relationship between ibandronate dose, changes in BMD, and rates of fractures. Lumbar spine BMD increased with increasing ibandronate dose and the incidence of fractures decreased as lumbar BMD increased. The pooled data pointed out the effectiveness of ibandronate to increase BMD and decrease fracture rate[44]. In our predominantly young CD patient setting, the increase in lumbar BMD with 1 mg 3-monthly iv dosing equaled the efficacy of ibandronate for the treatment of postmenopausal osteoporosis, and the overall increase in BMD in our CD patient setting was as good as with higher doses in postmenopausal osteoporosis[43,44].

When we planned this study, the discussion whether sodium fluoride can not only increase BMD but also prevent fractures was still open, and based on our pilot study we decided to have again a sodium fluoride intervention

arm. Here, the increase in lumbar BMD was somewhat less than in our pilot studies[32,45]. In both, serum fluoride at 0, 6 and 12 mo was in the effective range of 0.095-0.19 mg/L[46]. Nevertheless, the difference was most probably due to the lower sodium fluoride dose in the present study (50 mg *vs* 75 mg) which we chose based on an investigation using the same 50 mg dose and slow-release formula in postmenopausal women reporting an increase in BMD of 4%-5% per year[27]. There remains little information available on sodium fluoride and fracture rate and therefore the efficacy of sodium fluoride in preventing fractures remains controversial[27,28]. Nevertheless, Rubin has reported the efficacy of slow-release sodium fluoride in the prevention of vertebral fractures in postmenopausal osteoporosis[29]. In our study, only one incident vertebral fracture was diagnosed in the sodium fluoride group. With the scientific interest focused on bisphosphonates, this question will be left open and up to now, sodium fluoride is not approved for the treatment of osteoporosis, if any, in most countries..

To this day, some other studies have evaluated the management of osteoporosis in CD, most using bisphosphonates. The primary end-point in all these studies was BMD and none reported the prevalence and incidence of fractures. Haderslev *et al*[34] examined in a 12-mo double-blind, randomized, placebo-controlled trial the effect of 10 mg alendronate daily and reported a significant increase in lumbar BMD compared to placebo. Bartram *et al*[33]. reported an increase in BMD within 1 year with either a daily dose of 500 mg calcium and 400 IU vitamin D alone or with 3-monthly infusions of 30 mg pamidronate. The gain in BMD was a little more pronounced in the pamidronate group Siffledeen *et al*[35] reported a randomized trial of etidronate (400 mg orally) or not for 14 d and 500 mg calcium and 400 IU vitamin D for 76 d. This cycle was repeated 8 times. BMD significantly increased in both the etidronate- and the non-etidronate-treated groups.

Only a minority of recently diagnosed IBD patients had optimal serum 25-hydroxyvitamin-D_3 levels and serum 25-hydroxyvitamin-D_3 was positively correlated with baseline BMD of the lumbar spine, total hip, and total body, in a study by Leslie *et al*[13]. Therefore, optimization of vitamin D may play an important role in preventing IBD-related bone disease[13]. Vogelsang *et al*[30] prevented BMD loss in CD patients by long-term vitamin D supplementation. Increases in BMD were especially prevalent among patients who had normal serum levels of 25-hydroxyvitamin-D_3 (68%), whereas increases occurred in only 18% of patients with low serum levels of 25-hydroxyvitamin-D_3.

Our study in CD patients with reduced BMD (T-score < -1, i.e. osteopenia according to WHO criteria as published in 1994[36]) confirmed for the first time that the safe and well tolerated cholecalciferol and calcium supplementation alone not only prevented further bone loss but increased BMD of the lumbar spine for the better. Additional sodium fluoride or ibandronate had no benefit over cholecalciferol and calcium alone in managing reduced BMD. CD patients may take cholecalciferol and calcium first, and only add optional bisphosphonates, first and foremost in patients with reduced BMD and prevalent fractures, taking into account all the data on bisphosphonates and fracture rate in postmenopausal osteoporosis which we still do not have for CD. Our results support the common clinical practice reported with the implementation of the American College of Gastroenterology and American Gastroenterology Association osteoporosis screening guidelines in inflammatory bowel disease[47], with specific therapies based on DXA findings initiated in 69% of patients: oral calcium and vitamin D supplementation in 69% and bisphosphonates in 20%[48].

COMMENTS

Background

Reduced bone mineral density (BMD) commonly afflicts patients with Crohn's disease (CD). Many facts link the 2 states together. With reduced BMD, the fracture risk increases.

Research frontiers

In postmenopausal women, therapy for reduced BMD is well established, but not in CD. In postmenopausal women, the standard of care is bisphosphonates. In CD, this question is still open. In this study, the authors test the effectiveness and safety of basic cholecalciferol and calcium supplementation alone or along with oral sodium fluoride or intravenous ibandronate to improve BMD compared to baseline.

Innovations and breakthroughs

In this study, sodium fluoride or ibandronate had no added benefit over basic cholecalciferol and calcium supplementation alone in increasing BMD in patients with CD and reduced BMD at baseline. One vertebral fracture in the sodium fluoride group was not sufficient to suggest a difference between groups. The study medication was safe and well tolerated.

Applications

In CD patients with reduced BMD, cholecalciferol and calcium supplementation is common clinical practice. Our data support this approach to improve bone BMD in CD patients.

Peer review

This is an interesting paper for readers.

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BRIEF ARTICLE

T-regulatory lymphocytes in peripheral blood of gastric and colorectal cancer patients

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Abstract

AIM: To assess the absolute number of T-regulatory cells (Tregs; $CD4^+CD25^+Foxp3^+$) in the peripheral blood of gastric and colorectal cancer patients.

METHODS: We enrolled 70 cancer patients (33 gastric cancer, 37 colorectal cancer) and 17 healthy volunteers. The $CD3^+CD4^+$ lymphocytes and $CD4^+CD25^+Foxp3^+$ Tregs in the peripheral blood were analyzed with flow cytometry. The absolute numbers of Tregs were calculated based on the $CD4^+CD25^+Foxp3^+$ cells percentage of $CD3^+CD4^+$ cells and the absolute numbers of $CD3^+CD4^+$ cells per microliter.

RESULTS: The mean number of $CD4^+CD25^+Foxp3^+$ cells per microliter in colorectal cancer patients was 15.7 (SD: 21.8), for gastric cancer patients 12.2 (SD: 14.3), and for controls 17.5 (SD: 11.4). The absolute number of Tregs was significantly lower in gastric cancer patients than in controls (P = 0.026). There was no statistically significant difference for gastric *vs* colorectal cancer or colorectal cancer *vs* controls. The absolute number of Tregs was also significantly depressed in N^+ *vs* N^- cancer patients [22.0 (27.7) *vs* 10.1 (9.0), P = 0.013], and in the subgroup of gastric cancer patients [30.3 (27.6) *vs* 9.6 (8.0), P = 0.003]. No statistical difference was observed in the proportion of Tregs in the $CD4^+$ population between the groups.

CONCLUSION: The absolute number of Tregs in peripheral blood of gastric cancer but not colorectal cancer patients was significantly decreased in comparison with that in healthy controls.

Key words: $CD4^+CD25^+Foxp3^+$ cells; T regulatory cells; Peripheral blood; Gastric cancer; Colorectal cancer

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INTRODUCTION

There is evidence that regulatory T lymphocytes Tregs might be important for immunotolerance to self- and allo-antigens[1]. Activity of these cells is one of the mechanisms of immune evasion by tumors, which inhibits the antitumor activity of effector cells. They also suppress the antigen-presenting function of dendritic cells and the activity of natural killer cells[2,3]. Tregs are the subset of $CD4^+$ cells that express high levels of the interleukin-2 receptor α chain CD25. Therefore, Tregs are described as $CD4^+CD25^{++}$ cells. However, the value of CD25 as a specific marker is limited, because it is also expressed on activated $CD4^+$ cells. Recent studies have shown that Foxp3, a member of the transcription factor family, represents a specific marker for Tregs[4]. However, Foxp3 is a nuclear protein and it is impossible to use it for isolation of cells. Many previous studies on Tregs in neoplastic diseases that were performed before the discovery of Foxp3 used $CD4^+CD25^{++}$ subpopulation as an equivalent to Tregs. Recently, flow cytometric detection of $CD4^+CD25^+Foxp3^+$ in human cancer studies has been described[5]. The main outcome from studies in human cancer is the increase in the proportion of Tregs/$CD4^+$ cells among tumor-infiltrating lymphocytes (TILs), metastatic lymph nodes and peripheral blood[6]. The increase in Tregs was in some studies a prognostic factor of poor survival. However, these data are not uniform for all types and locations of tumors[7-9].

The aim of our study was to assess the absolute number of Tregs ($CD4^+CD25^+Foxp3^+$) in the peripheral blood of gastric and colorectal cancer patients.

MATERIALS AND METHODS

The study consisted of 70 patients (33 gastric cancer and 37 colorectal cancer) treated in a single institution between 2006 and 2009. All these patients had histologically confirmed disease. The median age was 68 years (range: 32-82 years). There were 42 male and 28 female patients. All these patients underwent laparotomy. The details of clinicopathological characteristics are summarized in Table 1.

None of the patients received chemotherapy, radiotherapy, immunotherapy or other form of therapy that influenced the immune system. Patients had no history of autoimmune disease or recent infection.

The blood of 17 healthy volunteers was tested as controls. The control group consisted of 10 men and seven women with a mean age of 42 years (range: 25-52 years).

The study was approved by the Ethical Committee of Jagiellonian University.

Blood samples were collected prior to any interventional procedure in sterile EDTA vacutainers. Peripheral blood samples (100 μL) obtained from cancer patients were incubated in TruCount tubes (BD Biosciences, San Jose, CA, USA) with a monoclonal antibody cocktail: FITC-conjugated anti-CD3 and PE-conjugated anti-CD4 (5 μL; BD Biosciences) for 30 min at 4℃. The samples were treated with 400 μL FACS Lysing Solution (BD Biosciences), and after erythrocyte lysis, 10000 $CD3^+CD4^+$ cells along with beads were acquired on a FACSCanto flow cytometer and analyzed with FACSDiva Software (BD Biosciences). The absolute numbers of $CD3^+CD4^+$ lymphocytes in samples were calculated on a basis of bead and lymphocyte counts. Tregs ($CD4^+CD25^+Foxp3^+$) were stained in 200 μL EDTA peripheral blood samples using the Human Regulatory T Cell Staining Kit (eBiosciences, UK), according to manufacturer's instructions, and acquired on the flow cytometer. The absolute numbers of Tregs were calculated based on the $CD4^+CD25^+Foxp3^+$ cells percentage of $CD3^+CD4^+$ cells and the absolute numbers of $CD3^+CD4^+$ cells per microliter.

Table 1 Clinicopathological characteristics

	No. of cases
Sex	
Male	42
Female	28
Age (yr)	
≤ 65	32
> 65	38
Tumor grade	
1	9
2 or 3	61
Lymph node metastases	
No	24
Yes	46
Distant metastases	
No	41
Yes	29
Stage (AJCC 2002)	
Ⅰ	13
Ⅱ	9
Ⅲ	13
Ⅳ	35

Statistical analysis

All quantitative variables were described as mean (SD). The Mann-Whitney *U* test and the χ^2 test were used when appropriate to compare distribution of individual variables between groups. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS version 14 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Absolute number of $CD4^+CD25^+Foxp3^+$ cells in peripheral blood

The mean number of $CD4^+CD25^+Foxp3^+$ cells per microliter in colorectal cancer patients was 15.7 (21.8), for gastric cancer patients 12.2 (14.3) and for controls 17.5 (11.4) (Figure 1).

The difference between colorectal cancer patients and the control group was not significant ($P = 0.079$). There was a significant difference between the gastric cancer patients and the control group ($P = 0.026$). The difference between the gastric cancer and colorectal cancer patients was not significant.

The absolute number of $CD4^+CD25^+Foxp3^+$ cells did not differ according to sex in either the colorectal or gastric

Table 2 Mean (SD) count of $CD4^+CD25^+Foxp3^+$ cells

	Colorectal cancer	P^1	Gastric cancer	P^1	Overall	P^1
Overall	15.7 (21.8)		12.2 (14.3)	0.449		
Sex		0.779		0.912		0.801
Male	16.9 (25.2)		11.3 (11.9)		14.4 (20.3)	
Female	13.5 (14.6)		13.4 (17.5)		13.5 (15.9)	
Age (yr)		0.849		0.080		0.309
≤ 65	18.7 (28.5)		10.3 (16.9)		15.0 (24.1)	
> 65	12.7 (12.2)		13.7 (12.4)		13.2 (12.2)	
Tumor grade		0.501	NA	1.000		0.865
1	16.8 (15.5)				14.9 (15.5)	
2 or 3	15.9 (24.8)				15.2 (21.9)	
Lymph node metastases		0.210		0.003		0.013
No	19.9 (28.1)		30.3 (27.6)		22.0 (27.7)	
Yes	11.0 (10.4)		9.6 (8.0)		10.1 (9.0)	
Distant metastases		0.596		0.882		0.447
No	17.6 (24.6)		15.8 (20.4)		17.0 (23.1)	
Yes	10.0 (7.8)		9.9 (8.3)		9.9 (8.0)	
Stage		0.267		0.195		0.088
Ⅰ or Ⅱ	19.9 (28.9)		33.4 (30.9)		22.4 (29.0)	
Ⅲ or Ⅳ	10.9 (10.4)		9.4 (7.8)		9.9 (8.9)	

[1]Mann-Whitney *U* test. NA: Not applicable.

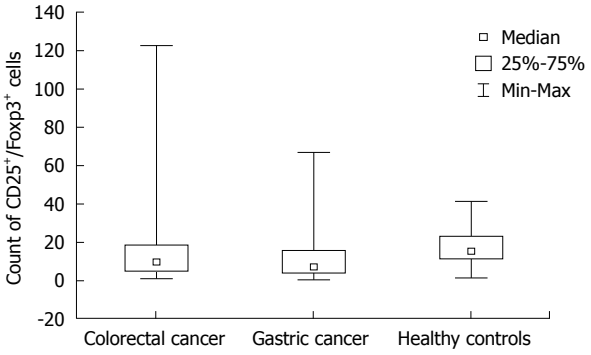

Figure 1 The absolute number of $CD4^+CD25^+Foxp3^+$ cells in peripheral blood of colorectal and gastric cancer patients and controls.

cancer patients. Patients aged ≤ 65 years and > 65 years had similar results for both cancer types (Table 2). The analysis of TNM stage revealed that, for more advanced-stage cancer, Treg count was lower but the difference was not significant.

In colorectal cancer patients, Treg absolute count was not related to tumor grade, lymph node status or distant metastases. There was also no difference between gastric cancer subgroups according to Lauren's histological classification. In gastric cancer patients, the number of $CD4^+CD25^+Foxp3^+$ cells was significantly lower in peripheral blood of N^+ patients ($P = 0.003$). For the pooled group of patients (gastric and colorectal cancer), this difference was also significant ($P = 0.013$). There was no difference between M^- an M^+ gastric cancer patients for absolute number of Tregs, or in the entire group of cancer patients.

Ratio of $CD4^+CD25^+Foxp3^+$ cells/$CD4^+$ cells in peripheral blood

The $CD4^+CD25^+Foxp3^+/CD4^+$ lymphocyte ratio did not show any differences between N^+ and N^- and M^+ and M^- patients for both types of cancer. This proportion was not related to sex or age (Table 3). There was no difference between stages Ⅰ / Ⅱ and Ⅲ / Ⅳ for either type of cancer, nor in the pooled group of cancer patients.

DISCUSSION

The prevalence of Tregs in various compartments in patients with tumors has been described as a potential prognostic factor. Tregs have been found in TILs (primary and metastatic tumors), in metastatic lymph nodes, malignant ascites, pleural effusion, and peripheral blood[6,10]. The prognostic significance of these findings is not uniform. Moreover, the impact of Tregs has been reported as their density in tumor stroma, proportion of TILs, Tregs/CD4 ratio, Tregs/CD3 ratio, or Tregs/CD8 ratio[11-14]. Mostly, the proportion of $CD25^+Foxp3^+$ cells among $CD4^+$ TILs has been analyzed.

Most of these studies were retrospective and based on the immunohistochemical examination of paraffin-embedded, previously collected specimens. On the other hand, peripheral blood is easily accessible and enables repeated measurements prior to surgery or in the follow-up period. Therefore, it is important to establish the pattern of Tregs in peripheral blood of patients with various tumors. However, the number of Tregs in peripheral blood changes less markedly than in TILs[11].

The present study was a pilot study, therefore, assessment of the number of cells was performed preoperatively only. The results of the assessment in the early post-operative period would probably be influenced by the pro-inflammatory and anti-inflammatory post-injury reactions. This might change the lymphocyte subpopulations. Our present results can form a background for subsequent studies on Tregs in the preoperative period and follow-up of cancer patients.

Table 3 Mean (SD) proportion of $CD4^+CD25^+Foxp3^+$ cells in the $CD4^+$ population (percentage)

	Colorectal cancer	P^1	Gastric cancer	P^1	Overall	P^1
Overall	2.18 (1.79)		2.01 (1.56)	0.760		
Sex		0.332		0.455		0.820
Male	2.31 (1.68)		1.89 (1.62)		2.12 (1.65)	
Female	1.98 (2.01)		2.18 (1.51)		2.08 (1.75)	
Age (yr)		0.456		0.610		0.777
≤ 65	2.32 (1.58)		1.88 (1.63)		2.13 (1.59)	
> 65	2.05 (2.00)		2.11 (1.54)		2.08 (1.76)	
Tumor grade		0.477		1.000		0.758
1	2.46 (1.72)		0		2.19 (1.80)	
2 or 3	2.11 (1.90)		2.10 (1.36)		2.09 (1.80)	
Lymph node metastases		0.316		0.388		0.172
No	2.60 (2.13)		2.73 (2.04)		2.63 (2.07)	
Yes	1.73 (1.25)		1.94 (1.53)		1.85 (1.41)	
Distant metastases		0.818		0.854		0.798
No	2.23 (1.98)		1.95 (1.72)		2.14 (1.88)	
Yes	2.03 (1.09)		2.05 (1.49)		2.04 (1.36)	
Stage		0.254		0.423		0.138
Ⅰ or Ⅱ	2.56 (2.22)		2.75 (2.35)		2.59 (2.18)	
Ⅲ or Ⅳ	1.66 (1.33)		1.66 (1.32)		1.66 (1.31)	

[1]Mann-Whitney *U* test. The Tregs/$CD4^+$ ratio in healthy volunteers group was 2.1%, and this was equal to that observed in gastric cancer patients.

In our study, the absolute number of $CD25^+Foxp3^+$ cells in peripheral blood did not differ between gastric and colorectal cancer patients. There was no difference between sex and age groups. Some authors have described increased prevalence of Tregs among T-lymphocyte populations in elderly patients[15]. Others have not observed this tendency[16]. There is also no clear evidence in the literature that the Tregs population is sex-related.

The absolute number of Tregs was significantly lower in gastric cancer patients than in controls. This is probably in contrast with other studies in gastric cancer patients, however, it is impossible to compare these results directly. The main problem is the difference in description of Tregs. Some early studies have analyzed the population of $CD4^+CD25^+$ cells, others $CD4^+CD25^{high}$ cells, and more recently, $CD4^+CD25^+Foxp3^+$ lymphocytes[12,13,17-20]. Most probably this is not the last word in the identification of functionally active Tregs. Even if these populations have a common core, they are not identical. Moreover, the technical process of identification can vary and bias the final result.

In colorectal cancer patients, the absolute number of Tregs in peripheral blood did not differ from that in healthy controls. There are not sufficient data to compare this result with other studies, because the main colorectal cancer studies have concentrated on prevalence of Tregs among TILs[12,13,21]. The pattern of Tregs localization and its correlation with tumor stage and prognosis also differs between gastric and colorectal cancer. The high prevalence of Tregs in colorectal cancer has been reported as a positive factor, contrary to gastric cancer for which it has been reported as a negative factor[7,8,11,13,22]. The reason for these differences might also be related to the different results observed in gastric and colorectal cancer patients in our study. However, the regulatory mechanisms of Treg maturation, activation and distribution are not fully understood. Therefore, we could not clarify the observed differences between the results in gastric and colorectal cancer.

We found that N^+ gastric cancer patients had significantly lower absolute counts of Tregs in peripheral blood than had N^- patients.

Our study included 25 node-positive patients and this probably influenced the mean Treg count in the whole group. Several studies have revealed an increase in Tregs in metastatic lymph nodes in gastric and esophageal cancer[23]. Our study might have demonstrated Treg migration to the metastatic lymph nodes and accumulation in the peritumoral infiltrate in more advanced tumors. There is some evidence that, in gastric cancer, Tregs might migrate to the tumor microenvironment *via* a chemokine-mediated mechanism[24].

The increase in Tregs among TILs has also been observed in colorectal cancer patients[25], but we did not observe a significant drop in peripheral blood Tregs in node-positive colorectal cancer patients. However, for the pooled N^+ *vs* N^- group, the difference was significant. The lack of difference between M^- and M^+ patients supports the hypothesis that the peripheral Tregs population does not change significantly during metastasis formation. The increase in $CD4^+CD25^{high}$ to $CD4^+$ cell ratio in comparison to that in healthy donors has been described in metastatic cancer[26]. However, the absolute numbers of Tregs in these populations have not been reported.

In our study, the number of patients was < 40 for each cancer type. These relatively small numbers preclude statistical analysis of Tregs counts at single TNM stages. Therefore, we performed our analysis on Ⅰ/Ⅱ *vs* Ⅲ/Ⅳ stages. The absolute number of Tregs was lower in the more advanced group, but the difference did not reach statistical significance.

The proportion of Tregs to $CD4^+$ cells was 2.18% for colorectal cancer and 2.01% for gastric cancer patients.

This value is located between that reported in the literature of 4%-6% for gastrointestinal cancer and 1%-3% for the healthy population[27]. However, the calculated percentages of Tregs (of CD4 or of CD3) or proportions of Tregs/CD8 is influenced by at least two variables. It should be considered that subpopulations of lymphocytes can change during tumor progression[28]. Therefore, the ratio and absolute numbers should be included.

In conclusion, our study was focused on the peripheral blood Tregs as a potential marker of disease, which was relatively easy to measure during the pretreatment and follow-up periods. The absolute number of Tregs in the peripheral blood of gastric cancer patients was significantly decreased in comparison to that in the healthy controls. This phenomenon was even strongly expressed in patients with lymph node metastasis. This was not observed in colorectal cancer patients. Our findings suggest that the population of Tregs in peripheral blood does not simply mimic stromal Tregs. Further studies on larger groups of patients are necessary to evaluate the Treg population in cancer patients.

COMMENTS

Background

The prevalence of T regulatory lymphocytes (Tregs) in various cancers has been described as a prognostic factor. The assessment of the absolute number of Tregs ($CD4^+CD25^+Foxp3^+$) in the peripheral blood of gastric and colorectal cancer patients can be used to monitor disease during treatment.

Research frontiers

Our study was focused on peripheral blood Tregs as a potential disease marker, which was relatively easy to measure during pretreatment and follow-up periods. The prevalence of Tregs in various compartments in patients with tumors has been described as a potential prognostic factor. Tregs have been found in tumor-infiltrated tissues and fluids, but the prognostic significance of these findings is not uniform. The impact of Tregs has been reported as their density in tumor stroma or the proportion of Tregs among tumor-infiltrating lymphocytes (TILs), Tregs/CD4 ratio, Tregs/CD3 ratio, or Tregs/CD8 ratio. However, overall, the proportion of $CD25^+Foxp3^+$ cells amongst $CD4^+$ TILs is analyzed.

Innovations and breakthroughs

Many studies of Tregs as a prognostic factor have been retrospective and based on immunohistochemical examination of paraffin-embedded, previously collected specimens. On the other hand, peripheral blood is easily accessible and enables repeated measurements prior to surgery or in the follow-up period. Therefore, it is important to establish the pattern of Tregs in peripheral blood of patients with various tumors.

Applications

In utilizing peripheral blood Tregs as a potential disease marker, this study demonstrates a way of improving the assessment and management of patients with gastric cancer.

Peer review

The authors assessed the absolute number of Tregs ($CD4^+CD25^+Foxp3^+$) in the peripheral blood of gastric and colorectal cancer patients. The absolute numbers of Tregs were calculated based on the $CD4^+CD25^+Foxp3^+$ cells percentage of $CD3^+CD4^+$ cells and the absolute numbers of $CD3^+CD4^+$ cells per microliter. The absolute number of Tregs in the peripheral blood of gastric cancer patients was significantly decreased in comparison to the healthy controls. This phenomenon was even strongly expressed in patients with lymph node metastasis, but not observed in colorectal cancer patients. The findings suggest that the population of Tregs in peripheral blood does not simply mimic stromal Tregs. Further studies on larger groups of patients are necessary to evaluate the Treg population in the blood of cancer patients.

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BRIEF ARTICLE

Detection of *Helicobacter pylori*: A faster urease test can save resources

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Abstract

AIM: To investigate whether differences in the rapidity of a positive result for *Helicobacter pylori* can save res ources, by comparing two commercially available urease kits.

METHODS: One hundred and eighty-five adults (130 outpatients, 55 inpatients) undergoing gastroscopy were entered prospectively. Patients were divided into two groups: Group 1 (if they were not on PPIs, antibiotics, H_2A, bismuth or sucralfate for up to 14 d prior to the endoscopy) and Group 2 (if they were on, or had been on, any of the above medication in the previous 14 d). At endoscopy two sets of biopsies, taken in random order, were placed in the wells of the *Campylobacter*-like organism (CLO) test (Kimberly-Clark, Utah, USA) and the Quick test (Biohit Plc, Helsinki, Finland). Five additional gastric biopsies were taken for histology/Giemsa and immunohistochemical study. The two urease test slides were read at 2 min, 30 min, 2 h and 24 h. Sensitivity and specificity at 24 h were determined.

RESULTS: At 24 h, for all patients, there was no difference in sensitivity (100% *vs* 97.5%), specificity (99.3%), positive (97.5%) and negative predictive values (100% *vs* 99.3%) between the CLO and Quick tests, respectively. There was a positive result at 30 min in 17/41 (41.5%) CLO tests, and in 28/40 (70%) Quick tests, $P = 0.05$. Quick test enabled the prescription of eradication therapy before discharge in all 28/40 patients. Only 12 (30%) follow-up appointments were needed. If the CLO test had been used alone, only 17 (41.5%) prescriptions would have been possible prior to discharge and 24 (58%) follow-up appointments would be needed ($P = 0.001$). Of 2000 gastroscopies performed annually at our unit, a saving of 123 follow-up appointments (total: 8856 Euros or 11 808 USD) would be achieved if we switched to the Quick test.

CONCLUSION: Direct comparison of locally available urease test kits is worthwhile, since the appropriate choice results in a significant saving of resources. Local costs and follow-up protocols will determine the magnitude of these savings.

Key words: *Campylobacter*-like organism test; Diagnosis; *Helicobacter pylori*; Quick test; Urease test kits

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped gram-negative bacterium which was identified in 1979[1]. It produces urease in abundance, the activity of which, through the production of ammonia, together with the bacterium's motility and ability to adhere to the gastric mucosa, enables its survival in the acid environment of the stomach. *H. pylori* is a causative agent for chronic active gastritis, peptic ulcer disease, gastric cancer and mucosa associated lymphoid tissue lymphoma[2]. It has also been shown to be associated with extragastric diseases, such as iron deficiency anemia and idiopathic thrombocytopenic purpura[3-5].

Non-invasive methods of *H. pylori* detection include serum antibody detection, fecal antigen tests[6] and the urea breath test[7]. Invasive methods of *H. pylori* detection require endoscopy in order to obtain gastric tissue for histologic determination, bacterial culture or for use in urease detection kits.

Urease detection kits are inexpensive and easy to use. Biopsies from the gastric mucosa are placed in a well containing a yellow colored agar gel which contains urea and a pH indicator. Urease cleaves urea liberating ammonia, which is alkaline turning the agar color red, so indicating the presence of a urea-producing organism. The test enables the determination of the *H. pylori* status of the patient within 24 h, with a substantial proportion giving a positive result within a few hours[8]. This represents a clear advantage over the costly and labor-intensive method of histological examination with special stain.

The aims of our study were to: (1) evaluate the sensitivity and specificity of two commercially available urease detection kits; (2) compare the time interval required for each kit to give a positive result; and (3) determine whether any differences would expedite patient management and save resources, by enabling treatment to be prescribed before patients are discharged from the endoscopy unit, thus avoiding a follow-up appointment.

MATERIALS AND METHODS

The study protocol was approved by the Hospital Ethics Committee. Patients over the age of 18 years referred for upper gastrointestinal endoscopy, in whom *H. pylori* detection was indicated, were enrolled prospectively, after written informed consent was obtained. Before gastroscopy, patients were asked whether they were, or had been in the previous 14 d, on treatment with proton pump inhibitors (PPIs), histamine type 2 receptor antagonists (H_2A), antibiotics, bismuth, or sucralfate. Patients not on PPIs, antibiotics, H_2A, bismuth or sucralfate for up to 14 d prior to the endoscopy, for the purpose of analysis were subsequently assigned to Group 1 and patients who were on, or had been on any of the above medication in the previous 14 d were assigned to Group 2. Patients on anticoagulants or with known prolonged international normalized ratio (INR), activated partial thromboplastin time (aPTT), or platelet count below 100 000/mL were excluded. Gastroscopy was performed routinely under light intravenous sedation and local anesthetic spray to the oropharynx.

The two urease detection kits used for comparison in this study were (1) the *campylobacter*-like organism (CLO) test Rapid Urease Test (Kimberly-Clark, Utah, USA), the gel of which contains urea United States Pharmacopeia (29 mg/mL), phenol red (a pH indicator), buffers and a bacteriostatic agent to prevent the growth of contaminating urease-positive organisms and (2) the *H. pylori* Quick test (Biohit Plc, Helsinki, Finland).

Both kits were kept at room temperature for at least 10 min prior to endoscopy. At endoscopy, two biopsy specimens, one from the antrum and one from the body (mid greater curve) of the stomach[7] were obtained for each urease test, each pair $\leqslant$ 1 cm apart. Each tissue pair was embedded in the same gel-containing well of the kits under investigation. Samples for the two urease tests were taken in a random order (sealed envelope). In each instance, following the biopsies for the urease tests, three biopsies from the antrum and two from the body of the stomach were obtained (within 1 cm of the previous biopsies) for histology/Giemsa and immunohistochemical staining. For each set of biopsies a new disposable spiked forceps with fenestrated cup was used (cup diameter 2.5 mm, Wilson Cook Medical Inc., Winston-Salem, NC, USA). The exact time of the placement of the biopsies in the urease test wells was recorded and the wells inspected for color change at 2 min, 30 min, 2 h and 24 h. The test was assigned positive when there was a color change of at least 2 mm radius of red cloud around the biopsy specimen, or complete color change of the yellow well to red or magenta.

Patients were discharged after 30-45 min post-endoscopy. Where a positive result was obtained, *H. pylori* eradication therapy was prescribed prior to discharge. The number of prescriptions issued before discharge was recorded. Patients not issued a prescription prior to discharge were given follow-up appointments for the result of the urease test and prescription of eradication therapy, where indicated. The financial burden of these extra appointments was calculated from data supplied by the accounts department of our hospital, comprising estimated administrative costs and cost of medical time.

Histology

The gastric mucosa tissue was fixed by a routine fixation system and was embedded in paraffin blocks. A series of three to four thick sections of each block were used for routine stains (hematoxylin/eosin-Giemsa) and immunohistochemistry. Immunohistochemical evaluation was performed as follows: de-paraffined sections of all blocks were pretreated in citrate buffer, pH 6.0 for 10-20 min followed by cooling at room temperature for 20 min. The primary antibody was then added (polyclonal rabbit anti-*H. pylori* serum at 1:250 dilution; Thermo Fischer Scientific, Runcorn, Cheshire, UK) and incubated for 30 min at room temperature. To detect antibody, a visualization system with diaminobenzene was used. Giemsa and immunostained slides were examined independently by two experienced histopathologists (Filippidis T and Leontara V)

Table 1 Sensitivity, specificity and predictive values for *Campylobacter*-like organism test and Quick test (all patients)

Test (at 24 h)	True positive	True negative	Total
CLO test			
Positive	40	1	41
Negative	0	144	144
Quick test			
Positive	39	1	40
Negative	1	144	145

Campylobacter-like organism (CLO) test: sensitivity 100% [95% confidence interval (CI): 91.24-100], specificity 99.3% (95% CI: 96.2-99.88), positive predictive value (PPV) 97.6% (95% CI: 87.4-99.57), negative predictive value (NPV) 100% (95% CI: 97.4-100); Quick test: sensitivity 97.5% (95% CI: 87.12-99.56), specificity 99.3% (95% CI: 96.2-99.88), PPV 97.5% (95% CI: 87.12-99.56), NPV 99.3% (95% CI: 96.2-99.88).

who were blind to the urease test results, using light microscopy; first separately and then their results were compared. Any differences were resolved by discussion between the two histopathologists. A true positive test was determined when any two of the four tests (CLO test, Quick test, Giemsa stain, immunohistochemical stain) were positive.

Statistical analysis

The sensitivity, specificity, positive and negative predictive values of the urease tests were determined for the overall number of the patients and separately for the group of patients not on PPIs, antibiotics, H_2A, bismuth or sucralfate for up to 14 d prior to the endoscopy (Group 1) and for the group of patients who were on, or had been on any of the above medication in the previous 14 d (Group 2).

Statistical comparison of the two urease tests was by the student *t*-test for two dependent proportions, χ^2 test and the McNemar test. A statistically significant difference in the comparison of the two kits was considered when *P* value was $\leqslant 0.05$. Confidence intervals (CI) were determined at the 95% level.

RESULTS

Sensitivity and specificity of CLO test and Quick test at 24 h - all patients

Between April and October 2007, 185 adult patients (101 male, 84 female); age range 18-82, mean 49 years, were entered into the study. One hundred and thirty were outpatients (70%) and 55 (30%) inpatients. The overall results were as follows.

CLO test was positive at 24 h in 41 cases (22%) and negative in 144 cases (78%). Quick test was positive at 24 h in 40 cases (22%) and negative in 145 cases (78%). Histology/Giemsa/immunohistochemistry was positive for *H. pylori* in 44 cases (23.8%).

For all 185 patients, the sensitivity, specificity, positive (PPV) and negative predictive value (NPV) for the CLO test and Quick test were similar (Table 1). The concordance of the CLO test and Quick test for a positive result was 95% and for a negative result was 98%.

Table 2 Sensitivity, specificity and predictive values of the *Campylobacter*-like organism and Quick tests in Group 1

Test (at 24 h)	True positive	True negative	Total
CLO test			
Positive	26	0	26
Negative	0	79	79
Quick test			
Positive	25	0	25
Negative	1	79	80

Campylobacter-like organism (CLO) test: sensitivity 100% [95% confidence interval (CI): 87.13-100], specificity 100% (95% CI: 95.36-100), positive predictive value (PPV) 100% (95% CI: 87.13-100), negative predictive value (NPV) 100% (95% CI: 95.36-100); Quick test: sensitivity 96.1% (95% CI: 81.11-99.32), specificity 100% (95% CI: 95.36-100), PPV 100% (95% CI: 86.68-100), NPV 98.7% (95% CI: 93.25-99.78).

Table 3 Sensitivity, specificity and predictive values of the *Campylobacter*-like organism and Quick tests in Group 2

Test (at 24 h)	True positive	True negative	Total
CLO test			
Positive	14	1	15
Negative	0	65	65
Quick test			
Positive	14	1	15
Negative	0	65	65

Campylobacter-like organism (CLO) test: sensitivity 100% [95% confidence interval (CI): 78.47-100], specificity 98.5% (95% CI: 91.9-99.73), positive predictive value (PPV) 93.3% (95% CI: 70.18-98.81), negative predictive value (NPV) 100% (95% CI: 94.42-100); Quick test: sensitivity 100% (95% CI: 78.47-100), specificity 98.5% (95% CI: 91.9-99.73), PPV 93.3% (95% CI: 70.18-98.81), NPV 100% (95% CI: 94.42-100).

Comparison of CLO and Quick test for patients on or off antisecretory drugs or antibiotics

Of the total 185 patients, Group 1 comprised 105 patients of whom 31 (29%) were inpatients. Group 2 comprised 80 patients of whom 24 (30%) were inpatients. None had been on bismuth or sucralfate. At 24 h, sensitivity, specificity, PPV and NPV was the same for the two kits, both for Group 1 and Group 2 (Tables 2 and 3).

Table 4 displays separately the results of the two urease test kits for Group 1 and 2. At 30 min, taking the CLO test and Quick test together, a total of 33 out of 51 tests were positive in Group 1, as compared to only 12 out of 30 in Group 2 ($P = 0.03$). At 2 h, there was no statistically significant difference between Group 1 and Group 2 ($P = 0.11$). In Group 1, 13 out of 26 CLO tests and 20 out of 25 Quick tests were positive at 30 min ($P = 0.02$), with no difference at 2 h. There was no statistically significant difference in the rapidity of the two urease tests at 30 min and 2 h in Group 2 ($P = 0.13$, $P = 0.14$, respectively).

Comparison of rapidity of a positive result for the CLO and Quick tests

The number of positive CLO and Quick tests for all patients at 2 min, 30 min, 2 h and 24 h is shown in Table 5. Of a total of 40 positive Quick tests at 24 h, only 12 re-

Table 4 Number of patients with a positive *Campylobacter*-like organism and Quick test at 2 min, 30 min, 2 h, 24 h for Groups 1 and 2

Time	Group 1		Group 2	
	CLO test	Quick test	CLO test	Quick test
2 min	2	5	0	3
30 min	13[a]	20[a]	4	8
2 h	23	25	11	14
24 h	26	25	15	15

[a]$P = 0.02$. CLO test: *Campylobacter*-like organism test.

Table 5 Number of patients with a positive *Campylobacter*-like organism and Quick test at 2 min, 30 min, 2 h, 24 h (all patients)

Time	CLO test	Quick test	*P*-value
2 min	2	8	0.03
30 min	17	28	0.05
2 h	34	39	0.28
24 h	41	40	0.45

CLO test: *Campylobacter*-like organism test.

mained negative at 30 min (30%), whereas 24 of a total of 41 positive CLO tests at 24 h (58%) remained negative at 30 min ($P = 0.001$). This enabled the prescription of *H. pylori* eradication therapy before departure from the endoscopy unit for 28/40 patients with a positive Quick test at 30 min and only 12 (30%) follow-up appointments were given.

Estimation of differences in financial costs and resources

Based on the above results if the CLO test had been used alone, only 17 (41.5%) prescriptions would have been possible ($P = 0.05$) prior to discharge and 24 (58%) follow-up appointments would be needed ($P = 0.001$). The additional financial cost of each of the additional 12 follow-up appointments at our hospital, for consultation and the prescription of eradication therapy, would be 17 Euros in administrative costs and 55 Euros in medical time (total: 72 Euros or 96 USD).

At our unit, just over 2000 gastroscopies are performed annually. Given our observed overall prevalence of *H. pylori* colonization of 41/185 (22%), we can expect 440 *H. pylori*-positive cases each year. Extrapolating from the data we present here on a difference of 28% in negative results at 30 min (58% CLO negative at 30 min *vs* 30% Quick negative), if the Quick test was used in preference to the CLO test, a saving of 123 follow-up appointments (total: 8856 Euros or 11 808 USD) would be achieved at our unit each year.

DISCUSSION

The diagnosis of *H. pylori* infection relies on various testing methods, with the gold standard being histology/staining[9]. Urease testing can provide rapid testing in the endoscopy suite, or in the hours following, but does not provide a gold standard assessment of infection.

We selected the CLO test and the Quick test for comparison because they were available locally. We considered that comparison of a greater number of urease test kits would not be justified due to the excessive number of gastric biopsies that this would entail.

Our results indicate, by using two biopsies placed in the same well, that there is no difference in the overall performance of the CLO test and Quick test at 24 h, with sensitivity at 100% and 97.5%, and specificity at 99%, respectively. There was, however, a significant difference in the rapidity of a positive test, in favor of the Quick test, which resulted in a significantly greater number of prescriptions issued prior to discharge at 30-45 min than would have been the case if the CLO test had been used alone.

Previous studies using similar methods also reported the sensitivity of the urease detection kits to be over 90%[10-12]. Goh *et al*[11] compared the HUITAI rapid urease test to histology and culture for *H. pylori* detection. Two biopsy specimens were used (antrum and body of stomach), as in our study. The sensitivity and specificity of the kits were 98.2% and 99%, respectively. In another study by Wong *et al*[12], the PyloriTek kit was evaluated using as gold standard histology and an in-house rapid urease test. In this study, only one biopsy from the antrum was used yielding 96.3% sensitivity and 97.9% specificity, and the benefit of the addition of a corpus biopsy was found to be marginal[12].

The results from the comparison of the reaction time of Groups 1 and 2 (Table 4) indicate that in patients with recent intake of antisecretory drugs or antibiotics the positivity of both urease tests is delayed at 30 min, although the final result at 24 h is not influenced. These findings are in agreement with those of van Keeken *et al*[10]. On the other hand, a decrease in sensitivity, in addition to delayed positivity, was reported by Prince *et al*[13], whilst Midolo *et al*[14] reported that false positive tests when acid suppression therapy is in use occur only after 24 h of incubation. The mechanism by which these medications interfere with the results is thought to be either by directly inhibiting *H. pylori* urease, or by changing the *H. pylori* colonization pattern[13].

There have been a number of previous comparisons of the speed of urease test kits: van Keeken *et al*[10] compared the accuracy and reaction time of a new dry rapid urease test, the GUT test, with the CLO test, culture and histology. The urease test was found reliable to read 60-120 min after endoscopy. Said *et al*[15] compared the accuracy and reaction time of a urease test, the Pronto Dry, with the CLO test and histology. A positive reaction time was achieved at 30 min, similar to the present study. In the study by Goh *et al*[11], the rapidity of the HUITAI rapid urease test was also examined. The median positive reaction time was 1.0 min (25%-75% inter-quartile range: 1.0-3.0 min); more rapid than that observed in the present study[11]. However, no data were given concerning the rapidity of the HUITAI test and its possible impact on resources. Caution should be exercised when

comparing studies of urease test reaction times in different populations, such as the European and Far Eastern. A crucial determinant of the rapidity of a positive urease test is the bacterial load present in the gastric biopsies. This may be higher in the Far East[16].

In our study, there was a significant difference in the rapidity of a positive urease result at 2 and 30 min after placement of the biopsies in the test wells (Table 5), in favor of the Quick test. As a result, we were able to prescribe *H. pylori* eradication therapy before discharge from the endoscopy unit in a significantly higher number of patients (so obviating the need for follow-up visit for the prescription of eradication therapy) than would have been the case if the CLO test had been used alone. The prevalence of *H. pylori* infection of 22% observed in our study is consistent with the 19% reported in a recent seroepidemiological study of Hellenic Navy recruits[17]. This rate is much lower than that reported in studies of the previous decade and is thought to be due to an improvement in lifestyle and socioeconomic status, in line with observations in other developed countries[16-18].

On the basis of our results we calculated that there would be a substantial annual saving in medical and administrative time as well as financial cost, if we adopted the Quick test in preference to the CLO test. In busier endoscopy units or areas of higher *H. pylori* prevalence, the benefit would be higher. Precise financial savings for each endoscopy unit would need to be calculated according to the outpatient follow-up protocol and to local costs. These vary widely between countries and institutions. Where the practice of endoscopy units is to delegate the reading of the urease test and prescription of eradication therapy to other providers, the cost saving would be transferred to the latter.

We conclude that in selecting from locally available urease test kits, direct comparison of the rapidity of a positive result is worthwhile because the appropriate choice of kit would result in a significant saving of resources.

COMMENTS

Background

Helicobacter pylori (*H. pylori*), is a urease (enzyme) producing organism responsible for chronic active gastritis, peptic ulcer disease, gastric cancer and mucosa associated lymphoid tissue lymphoma. One method of rapid detection is by the use of urease detection kits which are inexpensive and easy to use. These kits consist of a well containing a yellow colored agar gel; during gastroscopy, biopsies from the gastric mucosa are placed in the well. The presence of *H. pylori* will turn the agar color red, so indicating the presence of *H. pylori*. The test enables the determination of the *H. pylori* status of the patient within 24 h, and therefore the prescription of eradication therapy.

Research frontiers

According to the literature many urease detection kits have been studied for their sensitivity and specificity as well as for their rapidity. In this article, the authors emphasize the impact of the rapidity of the test on the financial and administrative costs.

Innovations and breakthroughs

The preferential use of a rapid urease test kit results in substantial annual savings in medical and administrative time as well as in financial cost.

Applications

Direct comparison of locally available commercial urease tests is worthwhile because it may lead to saving of resources.

Peer review

The diagnosis of *H. pylori* infection relies on various testing methods, with the gold standard being histology/staining. Urease testing can provide rapid testing in the endoscopy suite, or in the hours following, but does not provide a gold standard assessment of infection.

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BRIEF ARTICLE

Dietary zinc and metallothionein on small intestinal disaccharidases activity in mice

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Abstract

AIM: To examine the effect of increasing dietary zinc (Zn) intake and the lack of metallothionein (MT) expression on activity of small intestinal disaccharidases.

METHODS: MT-Ⅰ and Ⅱ knockout (MT-/-) and wild-type (MT+/+) female mice at 3.5 wk of age were randomly fed with a diet containing 2 (2 Zn), 15 (15 Zn) or 50 (50 Zn) mg Zn/kg (n = 8/group/genotype) for 5 wk. Small intestinal segments (duodenum, jejunum and ileum) were collected and either fixed in 10% formalin for histological analysis or snap frozen in liquid nitrogen for sucrase, lactase and maltase activity analyses.

RESULTS: Plasma Zn was significantly ($P < 0.05$) lower (33%) in MT-/- compared with MT+/+ mice fed the 2 Zn diet. Villus height and crypt depth were increased by approximately 15% in MT+/+ mice compared with MT-/- mice. Duodenal disaccharidase activities were significantly higher in MT+/+ compared with MT-/- mice particularly in those fed the 2 Zn diet. For the 50 Zn diet, jejunal sucrase and lactase activities were significantly higher in MT-/- (13313 ± 2314; 4107 ± 364 μmol glucose/well/min/g tissue, respectively) compared with MT+/+ mice (7054 ± 608; 1818 ± 174). Similarly, ileal lactase activities were higher in MT-/- (1480 ± 192) compared with MT+/+ (629 ± 353) mice particularly those fed the 2 Zn diet.

CONCLUSION: Increasing dietary Zn has little effect on disaccharidases activity in MT wild-type mice. The presence of MT may enhance morphological and functional development of the gut.

Key words: Lactase; Maltase; Sucrase; Metallothionein; Diet; Zinc

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INTRODUCTION

Gastrointestinal function is characterized by growth, structural and functional changes of the crypts and villi, macro-

molecular absorption capacity and alteration in the activity of the small intestinal brush-border disaccharidases[1]. It is well documented that weaning is associated with marked changes in the histology and biochemistry of the small intestine, and such changes include increased cell proliferation and differentiation[2] and altered activity of the brush border disaccharidases, lactase and sucrase[1]. These changes are often used as indicators of small intestinal maturity and development following the consumption of a solid diet.

The brush border disaccharidases, lactase and sucrase, are considered accurate markers of enterocyte maturity and functional capacity[3]. It is well documented that dietary modification is correlated with significant changes in the histology and biochemistry of the small intestine[4]. Furthermore, pigs fed an inorganic zinc (Zn) diet and weaned pigs from sows fed an inorganic Zn diet compared with those fed the control diet had improved gut morphology[5,6]. In addition, Zn deficiency has been shown to reduce villous dimensions and increase villous density in the small intestine; however after a short period of Zn supplementation, villous density basal width and the maximum height of individual villi returned to normal[7]. Duff and Ettarh[8] demonstrated that Zn-fed mice produced more crypt cells in the distal part of small intestine compared with controls. Furthermore, it has been shown that weanling rats fed a semi-purified Zn-deficient diet have significantly lower levels of sucrase, maltase, lactase, leucine aminopeptidase, and alkaline phosphatase compared with rats fed a control or high Zn diet[9,10].

Zn is an essential nutrient required for cell growth, differentiation, and survival, and its deficiency causes growth retardation, immunodeficiency, and other health problems[11]. Therefore, Zn homeostasis must be tightly controlled in individual cells. The transcellular uptake of Zn occurs in the distal duodenum and proximal jejunum[12-15] from the brush border membrane. The mechanisms of exogenous zinc uptake have not yet been entirely elucidated, although both saturable and nonsaturable processes are involved[16]. Zn has been shown to be a potent inducer of its endogenous binding protein, metallothionein (MT)[17,18], a low molecular weight, intracellular cysteine-rich, metal-binding protein that consists of 4 isoforms (MT-1 to MT-4), with MT-1 and MT-2 being the most widely expressed isoforms[19]. MT is found mainly in the liver, kidneys, intestine and pancreas[17]. MT synthesis is induced by a number of metals, cytokines and stress hormones as well as by a wide range of chemicals, many of which act indirectly *via* a stress or inflammatory response[20,21].

Metal regulation of MT genes has been covered in several recent reviews[22,23]. Briefly, the binding of Zn to the metal transcription factor-1 allows the protein to bind to metal response elements in the promoter region which, in turn, initiates MT-gene transcription. Functions of MT include protection from cell apoptosis, promotion of cell proliferation and differentiation, regulation of Zn pools in circulation and in cells, scavenging of free radicals and protection against toxicity of heavy metals[17-19]. We hypothesized that an increase in dietary Zn and a lack of MT-1 and MT-2 expression will alter small intestinal disaccharidases activity. Thus the aims of the present study were to investigate the effects of various concentrations of dietary Zn intake (2, 15, or 50 mg Zn/kg diet) and the lack of MT-1 and MT-2 expression on small intestinal morphology and activity of disaccharidases in mice. This is applicable to the young infant starting complementary feeding whether dietary Zn may aid in nutrient absorption.

MATERIALS AND METHODS

Animals

Twenty four MT wild-type (MT+/+) C57BL/6 mice were obtained from the University of Adelaide (Adelaide, South Australia) and 24 MT-1 and 2 null (MT-/-) mice were obtained from a breeding colony at the Children, Youth and Women's Health Service Animal Care Facility (North Adelaide, South Australia). MT-/- (mixed genetic background of OLA129 and C57BL6 strains) mice were F3 derivatives of the interbreeding of normal C57BL6 mice[24].

At 3 wk old, mice were randomly allocated to be fed with either a 2, 15 or 50 mg Zn/kg diet (2 Zn, 15 Zn and 50 Zn) for 5 wk (n = 8/group/genotype) and body weight was recorded weekly. Mice were fed a casein-based diet[25] supplemented with $ZnSO_4$ to 2, 15 or 50 mg Zn/kg. The casein-based diet contained (g/kg): cornflour starch, 514; casein, 180; sucrose, 152; wheat bran, 50; peanut oil, 50; D,L-methionine, 2.5; choline chloride, 1 and codliver oil, 4.4. The mineral profile (g/kg diet) was: KH_2PO_4, 17.155; $CaCO_3$ 14.645; NaCl, 12.530; $MgSO_4 \cdot 7H_2O$, 4.99; $FeC_6H_5O_7 \cdot 5H_2O$, 0.296; $CaPO_4$, 0.170; $MnSO_4 \cdot 4H_2O$, 0.080; $CuSO_4$, 0.123; KI, 0.00025; $(NH_4)_6Mo_7 \cdot O_{24} \cdot 4H_2O$, 0.00125; Na_2SeO_3, 0.00005. The vitamin profile (mg/kg diet) was: thiamine HCl, 70; riboflavin, 30; niacin (nicotinic acid), 50; pantothenic acid, 150; pyridoxal HCl, 15; hydroxycobalamin, 0.02; inositol, 400; ρ-aminobenzoic acid, 50; folic acid, 10; biotin, 0.4 and glucose, 225. For the purpose of this study we chose the 15 mg Zn/kg diet as the normal (control) diet and the 2 and 50 mg Zn/kg diet as the low and high Zn diet respectively. The Zn diets were given to the animals *ad libitum* and the Zn content of the diets was validated by atomic absorption spectrophotometry using a Perkin-Elmer 3030 (Überlingen, Germany).

Tissue collection

At the end of the experimental period, all mice were CO_2 asphyxiated and blood was withdrawn by cardiac puncture. The mice were then killed by cervical dislocation. The liver and gut were excised and the pancreas and mesentery removed. The gut was separated into stomach, small intestine, cecum and colon. The contents of the small intestine were flushed thoroughly with saline. The small intestine was then divided into the duodenum, from the gastro-duodenal junction to the ligament of Treitz, and into two segments of equal length comprising the jejunum, and ileum. A 4 cm segment of the duodenum, jejunum and ileum were excised for histological assess-

ment and disaccharidase activity analysis, respectively. The protocol adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approval was obtained from the Animal Care and Ethics Committee of the Women's and Children's Hospital (South Australia).

Plasma Zn

Blood samples were placed in lithium heparin tubes to obtain plasma, which was stored at -20℃ until analysis. Plasma samples were diluted (1:2) in 10% trichloroacetic acid (Sigma-Aldrich, Sydney Australia) and were analyzed for Zn concentration (μmol/L) by atomic absorption spectrophotometry using a Perkin-Elmer 3030 (Perkin-Elmer Pty Ltd, Uberlingen, Germany).

Histological assessment

Small intestinal segments of 2 cm were removed and fixed in 10% formalin overnight, processed and embedded in paraffin wax (cross section), from which 4 μm-thick small intestinal sections were cut using the RM2235 microtome (Leica, Germany) and mounted onto glass slides. Sections were dewaxed and stained with Lilie-Mayer's hematoxylin and eosin and coverslipped. On each section, approximately 40 crypt depths and villi heights (expressed as μm) were measured using the Eclipse50i light microscope (Nikon, Japan) and Image ProPlus 5.0 package (Media Cybernetics, USA).

Disaccharidase assays

Disaccharidase activities in intestinal segments were assessed using a microplate modification of the Dahlqvist[26] assay in duplicates. Gut segments were homogenized in 10 mmol/L phosphate-buffered saline by Ultra-TurraxT25 homogenizer (Janke and Kunskel, Germany). Homogenates were centrifuged at 3500 rpm at 4℃ for 10 min. The supernatant was then aliquoted and snap-frozen at -80℃ for subsequent sucrase, maltase and lactase activity assays. Glucose standards (0, 1.25, 2.5, 5, 10, 20, 30 and 40 nmol/L) were prepared. Samples were diluted 1:20, 1:50 and 1:100 with 50 mmol/L phosphate buffer and then pipetted onto a 96-well plate. A solution of 0.2 mol/L sucrose, lactose or 0.004 mol/L maltose was added (50 μL/well) and the plate incubated at 37℃ for 30 min. Tris-glucose oxidase was added to all wells and incubated at 37℃ for another 30 min. Absorbance was determined using a Tecan spectrophotometer (Sunrise, Austria) set at 490 nm wavelength. Disaccharidase activities were analyzed using TableCurve (Systat Software, USA) and results were expressed as μmol glucose/well/min/g of tissue.

Statistical analysis

Histological analysis data were not normally distributed therefore data were log transformed and are presented as geometric mean ± SE of the mean. Disaccharidase activity data are expressed as mean ± SE of the mean. All data were analyzed using two-way analysis of variance followed by Tukey's *post-hoc* test (SigmaStats3.0). The significance level was determined as $P < 0.05$.

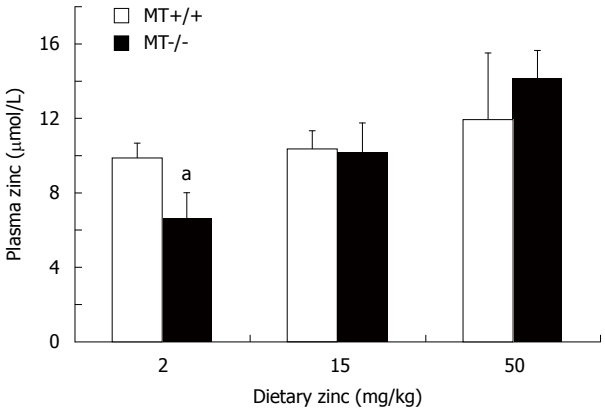

Figure 1 Comparison of plasma zinc (mean ± SE of the mean) of MT+/+ and MT-/- mice fed the 2, 15 and 50 mg/kg Zn diet for 5 wk (*n* = 8/group/genotype). [a]*P* < 0.05 *vs* MT+/+ mice fed the 2 mg/kg Zn diet. MT: Metallothionein.

RESULTS

Body weight and plasma Zn

There were no differences in body weights of both MT+/+ and MT-/- mice between the dietary Zn groups (data not shown). There were no differences in plasma Zn levels between MT+/+ and MT-/- mice when fed the 15 Zn or 50 Zn diet. However, there was a significant ($P < 0.05$) decrease (33%) in plasma Zn in MT-/- mice fed the 2 Zn diet compared with MT+/+ counterparts (Figure 1). There was an apparent trend of an increase in plasma Zn levels as the dietary Zn levels increased in MT-/- mice. Whereas in MT+/+ mice this was more tightly controlled with no difference in plasma Zn with increasing dietary Zn.

Small intestinal weight and length

An increase in dietary Zn did not change small intestinal weight between MT+/+ and MT-/- mice. However, there was a significant ($P < 0.05$) difference in small intestinal weight in MT-/- mice fed 2 Zn (0.74 ± 0.04 g) and 50 Zn (0.70 ± 0.03 g) compared with those fed the 15 Zn (0.59 ± 0.02 g). Interestingly, MT-/- mice had a significantly ($P < 0.05$) longer small intestine compared with MT+/+ mice, in particular in mice with the 2 Zn (29.8 ± 0.8 cm *vs* 25.7 ± 0.9 cm, respectively) and 50 Zn diets (29.1 ± 0.6 cm *vs* 26.8 ± 0.6 cm, respectively).

Villus height and crypt depth

Histological analysis (Tables 1 and 2) of the small intestine in MT+/+ and MT-/- mice showed that increasing dietary Zn concentrations did not alter villus height and crypt depth ($P > 0.05$). Differences in ileal villus height were insignificant irrespective of genotypes and dietary groups (Table 1). However, shorter villi were observed in the duodenum and jejunum of MT-/- mice fed with 15 Zn and/or 50 Zn diet(s) compared with MT+/+ mice (Table 1). Crypt depth was also significantly shorter in the duodenum (all dietary groups), jejunum (15 Zn and 50 Zn) and ileum (2 Zn) in MT-/- mice ($P < 0.05$) (Table 2).

Disaccharidase activities

MT-/- mice fed the 2 Zn diet had a significantly reduced

Table 1 Villus height (μm) in MT+/+ and MT-/- mice fed the 2, 15 or 50 mg/kg Zn diet for 5 wk

Intestinal segments	Dietary Zn intake (mg/kg)	Genotype	
		MT+/+	MT-/-
Duodenum	2	0.47 ± 0.03	0.37 ± 0.05
	15	0.42 ± 0.03	0.42 ± 0.03
	50	0.50 ± 0.03[a]	0.34 ± 0.03
Jejunum	2	0.42 ± 0.03	0.45 ± 0.03
	15	0.44 ± 0.03[a]	0.34 ± 0.04
	50	0.48 ± 0.03[a]	0.38 ± 0.04
Ileum	2	0.21 ± 0.01	0.22 ± 0.02
	15	0.20 ± 0.01	0.24 ± 0.02
	50	0.22 ± 0.04	0.21 ± 0.02

[a]$P < 0.05$ *vs* MT-/- mice. Data are expressed as mean ± SE of the mean (n = 8/diet/genotype). MT: Metallothionein.

Table 2 Crypt depth (μm) in MT+/+ and MT-/- mice fed the 2, 15 or 50 mg/kg Zn diet for 5 wk

Intestinal segments	Dietary Zn intake (mg/kg)	Genotype	
		MT+/+	MT-/-
Duodenum	2	0.099 ± 0.006[a]	0.069 ± 0.004
	15	0.182 ± 0.014[a]	0.069 ± 0.005
	50	0.104 ± 0.004[a]	0.085 ± 0.009
Jejunum	2	0.086 ± 0.004	0.080 ± 0.003
	15	0.084 ± 0.003[a]	0.066 ± 0.004
	50	0.093 ± 0.003[a]	0.077 ± 0.003
Ileum	2	0.091 ± 0.004[a]	0.077 ± 0.005
	15	0.087 ± 0.006	0.075 ± 0.004
	50	0.085 ± 0.004	0.075 ± 0.004

[a]$P < 0.05$ *vs* MT-/- mice. Data are expressed as mean ± SE of the mean (n = 8/diet/genotype). MT: Metallothionein.

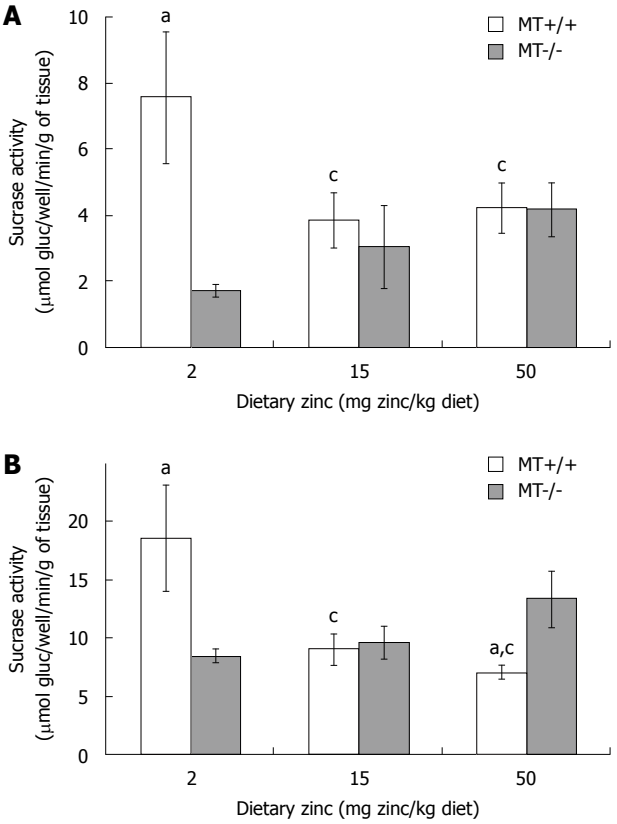

Figure 2 Comparison of sucrase activity in the duodenum (A) and jejunum (B) of MT+/+ and MT-/- mice fed the 2, 15 and 50 mg/kg Zn diet for 5 wk (n = 8/group/genotype). [a]$P < 0.05$ *vs* MT-/- mice fed the 2 or 50 mg/kg Zn diet, respectively; [c]$P < 0.05$ *vs* MT+/+ mice fed the 2 mg/kg Zn diet. MT: Metallothionein.

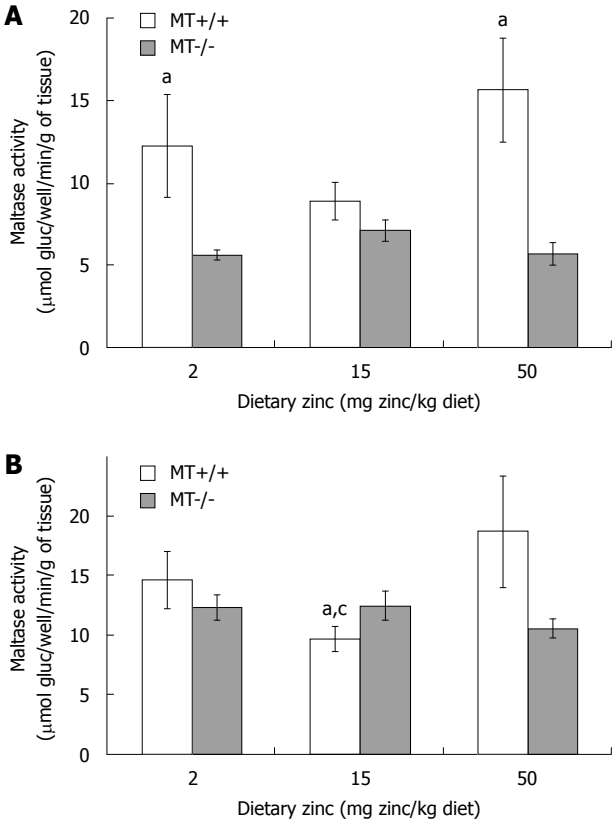

Figure 3 Comparison of maltase activity in the duodenum (A) and jejunum (B) of MT+/+ and MT-/- mice fed the 2, 15 and 50 mg/kg Zn diet for 5 wk (n = 8/group/genotype). [a]$P < 0.05$ *vs* MT-/- mice fed the 2, 15 or 50 mg/kg Zn diet, respectively; [c]$P < 0.05$ *vs* MT+/+ mice fed the 50 mg/kg Zn diet. MT: Metallothionein.

duodenal and jejunal sucrase activity compared with MT+/+ mice (Figure 2). Interestingly, sucrase levels were significantly lowered in the jejunum of MT+/+ mice receiving 15 Zn and 50 Zn (by 12%) compared with 2 Zn (Figure 2B). MT+/+ mice fed with 50 Zn also showed a lower activity in the jejunum compared with MT-/- mice. Results in both figures showed a trend of a decrease in sucrase activity in MT+/+ mice and an increase in MT-/- mice as dietary Zn concentration increased ($P < 0.05$). No significant differences were obtained for the ileum regardless of dietary Zn and genotypes ($P > 0.05$).

Duodenal maltase activity of MT+/+ mice fed with 2 Zn and 50 Zn was 37% and 47% higher, respectively, than that of MT-/- mice receiving same levels of dietary Zn (Figure 3A). When dietary Zn intake was increased from 15 Zn to 50 Zn, MT+/+ mice exhibited a marked increase in jejunal maltase activity (Figure 3B). Significant differences were also observed between MT+/+ and MT-/- mice receiving 15 Zn ($P < 0.05$). No differences were seen in ileal maltase activity regardless of dietary Zn and genotypes ($P > 0.05$).

In MT+/+ mice, there was a trend of a decrease in

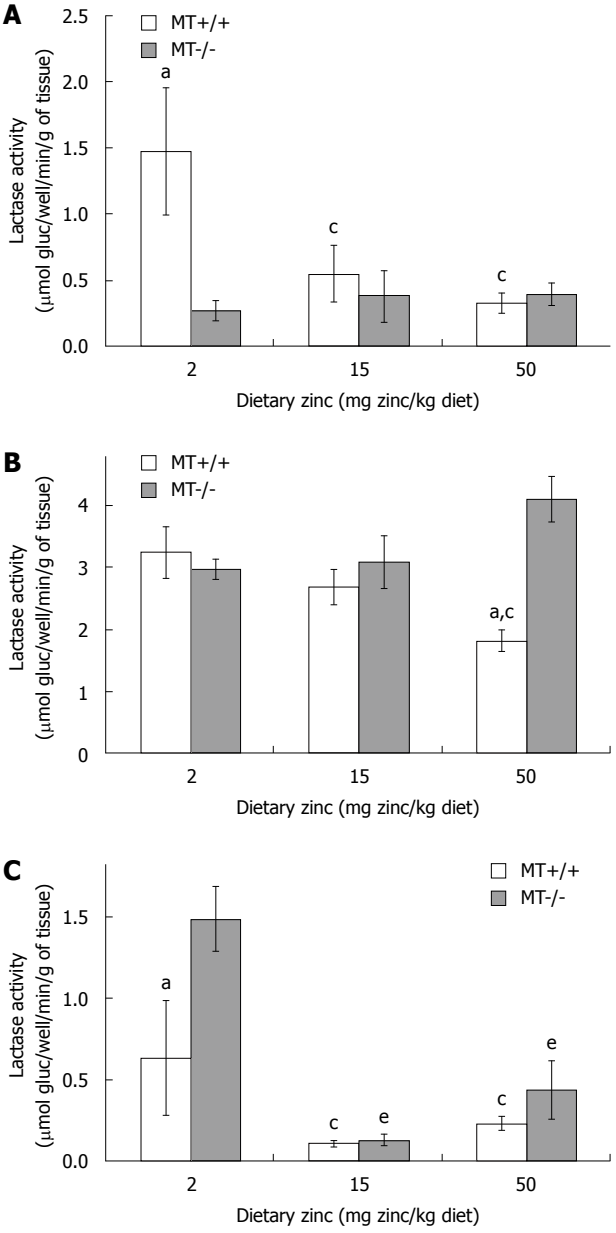

Figure 4 Comparison of lactase activity in the duodenum (A), jejunum (B) and ileum (C) of MT+/+ and MT-/- mice fed the 2, 15 and 50 mg/kg Zn diet for 5 wk (*n* = 8/group/genotype). [a]*P* < 0.05 *vs* MT-/- mice fed the 2 or 50 mg/kg Zn diet; [c]*P* < 0.05 *vs* MT+/+ mice fed the 2 mg/kg Zn diet; [e]*P* < 0.05 *vs* MT-/- mice fed the 2 mg/kg Zn diet. MT: Metallothionein.

lactase activity when the dietary Zn levels increased in different regions of the small intestine (Figure 4A-C). For example, MT+/+ mice receiving 50 Zn showed 28% reduction ($P < 0.05$) in jejunal lactase activity compared with the 2 Zn group (Figure 4B). Interestingly, in MT-/- mice, while the increase in dietary Zn levels did not appear to alter the lactase activity in the duodenum (Figure 4A) and in the jejunum (Figure 4B), the ileal activity was significantly lower in the 15 Zn and 50 Zn groups by 84% and 55%, respectively, compared with MT-/- mice fed with 2 Zn (Figure 4C). Genotype comparisons showed that, in MT-/- mice, the lactase activity in the duodenum was significantly lower when fed the low Zn diet (2 Zn, Figure 4A), but was significantly higher ($P < 0.05$) in the jejunum when fed the high Zn diet (50 Zn, Figure 4B) and in the ileum when fed the low Zn diet (2 Zn, Figure 4C), compared with their MT+/+ counterparts.

DISCUSSION

The development profile of disaccharidase activity in rodents seems to be well correlated with the transition from milk to solid food consumption[1]. Although it has been reported that dietary modification is associated with altered activity of the brush border disaccharidases[6,8,27], the influence of dietary Zn levels and the interaction of dietary Zn with its endogenous binding protein MT on small intestinal maturation has been unclear. Thus, in the present study, we examined the effect of increasing dietary Zn concentration and the role of MT-1 and 2 gene knockout on morphological changes and disaccharidase levels in weanling mice. To our knowledge, this is the first study to assess Zn supplementation on small intestinal morphology in wild-type and MT-1 and 2 null mice.

The measurements of villus height and crypt depth provide an indication of the maturity and functional capacity of small intestinal enterocytes. In the present study, although we have shown that increasing dietary Zn intake did not appear to significantly increase growth of the small intestine morphologically, as demonstrated in the histological analysis of villus height and crypt depth, the MT+/+ mice fed the high Zn diet had a higher villus height in most of the regions of the small bowel. This is consistent with a previous study[6], showing villus height in the jejunum of sows fed Zn was greater than those fed the control diet. Similar trends were observed in other studies showing that, although not significant, pigs supplemented with Zn tend to have an increased villus height:crypt depth ratio in the jejunum and higher goblet cell counts in the ileum[5,28]. It has been speculated that the differences in the intestinal morphology of pigs are being masked because of deterioration in the mucosa immediately after weaning, a common phenomenon in weaning pigs. However, growth performance was improved by the addition of ZnO which responded linearly to incremental doses of Zn[28]. In our current study, we further showed that the presence of MT markedly increases villus height and crypt depth in the small intestine, in particular in mice fed a high dietary Zn, and that crypt depth is generally greater in the wild-type mice than the null mice, suggesting the importance of MT- I and II in supporting optimal intestinal growth in response to dietary Zn supply.

Considering the role of Zn as a coenzyme for more than 300 enzymes particularly in RNA-DNA synthesis and cell proliferation[11], these improvements in intestinal morphology may be explained by the beneficial effects of Zn on cell proliferation, differentiation, and protein synthesis[29]. This is supported by the trend of increase in plasma Zn with increasing dietary Zn in the present study and is consistent with our previous study[30]. In contrast, Zn deficiency has been shown to cause villus atrophy, elevated levels of mucosal cell apoptosis, ulceration, inflammation as well as reduction in crypts proliferation[8].

Furthermore, it has been reported that Zn deficiency may impair carbohydrate digestion, as reflected in decreased disaccharidase activities, and may contribute to poor nutrition[10]. The results of these findings suggest that dietary addition of Zn in the normal diet of mice is vital for small intestinal mucosal integrity as well as improved small intestinal morphology.

In general, intestinal disaccharidase activities are lower in MT-/- compared with MT+/+ mice particularly at the low dietary Zn concentration (except for jejunal sucrase and lactase activity in the ileum), indicating that the combination of low dietary Zn and the lack of MT expression have a negative impact on the activity of disaccharidases in the small intestine. MT is a binding protein that regulates the quantity of Zn absorbed by binding Zn within the mucosal cells, thereby regulating its transfer across the basolateral membrane into the circulation and deposition in the liver[17,31]. We have shown that MT-/- mice accumulate less Zn[25], whereas transgenic mice accumulate more[19]. This is consistent with our data where MT-/- mice have lowered plasma Zn compared with MT+/+ mice, in particular with low dietary Zn intake, suggesting that the presence of MT- Ⅰ and Ⅱ plays a role in maintaining Zn homeostasis at low dietary Zn intake. It is possible that the low Zn status may be a contributing factor to decreased levels of disaccharidase activities in MT-/- mice. This is consistent with other studies[7,9,10,32] which reported that Zn deficiency causes marked reductions in intestinal mucosal protein content and disaccharidase activity. Furthermore, MT has been shown to have metalloregulatory functions in cellular growth and differentiation[33], and the lack of MT- Ⅰ and MT- Ⅱ expression may also be another contributing factor to the lower levels of disaccharidase activities in MT-/- mice.

Interestingly, increasing dietary Zn in the diet did not have a significant increase on intestinal disaccharidase activity in the wild-type mice. This is consistent with previous studies[7,34] where Zn supplementation has little or no effect on brush border disaccharidases. We have shown that increasing the dietary Zn concentration to 50 mg Zn/kg diet increases maltase activity only. It is possible that the highest dietary Zn concentration in the present study may be too low to induce disaccharidase activity.

MT has long been implicated in the regulation of absorption and excretion of Zn by the intestine[31,35] and the presence of MT has been shown to restrict Zn absorption at high Zn concentrations[12]. It has been argued that MT limits Zn absorption by sequestering it in the intestinal wall, thereby transiently reducing its absorption and favoring Zn transfer back into the gut lumen[12,14]. Furthermore, it has been shown that at high Zn concentration, MT-/- mice absorb Zn more readily and retain more Zn in the intestinal wall compared to MT-transgenic mice[36]. The high Zn intakes may explain why MT-/- mice have significantly greater jejunal sucrase and lactase activities than MT+/+ mice by inducing small intestinal disaccharidase activities. These adaptive mechanisms play a central role in nutrient processing and absorption which maintain normal homeostasis in these mice. This is also consistent with MT-/- mice having a heavier and longer small intestine, an adaptive mechanism to absorb more Zn to maintain Zn homeostasis when dietary Zn is high. These changes in intestinal disaccharidase activity in response to the interaction of dietary Zn and endogenous MT remain to be investigated. It is possible that intestinal disaccharidase activity and MT expression may not be related. To our knowledge this is the first study to report intestinal brush border enzyme activities in MT-/- mice. Thus, the role of MT in the expression of disaccharidase activities, particularly in MT-/- mice, warrants further investigation.

In conclusion, limiting the level of dietary Zn in the diet does not affect the activity of small intestinal brush border disaccharidases in wild-type mice. However, the presence of MT enhances the morphological and functional development of the gastrointestinal tract. Thus, simple Zn supplementation may be insufficient to drive growth and development of the small intestine. In light of the present findings, future studies investigating the close interaction between Zn and MT on gut maturation and development are warranted.

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COMMENTS

Background

The development of brush border enzymes and gut maturation is associated with complementary feeding. However, it is unclear whether the activity of the brush border disaccharidases is influenced by dietary zinc levels and the presence of the endogenous binding protein metallothionein.

Research frontiers

The role of metallothionein on gut maturation and development, in particular, in small intestinal morphology and brush border enzymes is an important area of research in the future.

Innovations and breakthroughs

The present study demonstrated that the presence of metallothionien, a zinc binding protein, may have a positive impact on villous morphology and digestive and absorptive function of the small intestine.

Applications

Future studies investigating the role of different levels of metallothionein in the gut on small intestinal morphology and the activities of brush border enzymes which may elucidate the underlying mechanism of metallothionein in altering intestinal physiology, villous architecture, and enzyme activities.

Terminology

Disaccharidases - enzymes located on the brush border and which are essential for digestion and absorption.

Peer review

Tran *et al* report on a basic research study in metallothionein wild-type and knockout mice testing the influence of different amounts of alimentary zinc on small intestinal disaccharidases. The paper is well written and the point is clearly made.

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BRIEF ARTICLE

Intrahepatic biliary cystic neoplasms: Surgical results of 9 patients and literature review

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Abstract

AIM: To investigate the eligible management of the cystic neplasms of the liver.

METHODS: The charts of 9 patients who underwent surgery for intrahepatic biliary cystic liver neoplasms between 2003 and 2008 were reviewed retrospectively. Informed consent was obtained from the patients and approval was obtained from the designated review board of the institution.

RESULTS: All patients were female with a median (range) age of 49 (27-60 years). The most frequent symptom was abdominal pain in 6 of the patients. Four patients had undergone previous laparotomy (with other diagnoses) which resulted in incomplete surgery or recurrences. Liver resection (n = 6) or enucleation (n = 3) was performed. The final diagnosis was intrahepatic biliary cystadenoma in 8 patients and cystadenocarcinoma in 1 patient. All symptoms resolved after surgery. There has been no recurrence during a median (range) 31 (7-72) mo of follow up.

CONCLUSION: In spite of the improvement in imaging modalities and increasing recognition of biliary cystadenoma and cystadenocarcinoma, accurate preoperative diagnosis may be difficult. Complete surgical removal (liver resection or enucleation) of these lesions yields satisfying long-term results.

Key words: Biliary cystadenoma; Cystadenocarcinoma; Enucleation; Hepatic resection

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INTRODUCTION

The first account of an intrahepatic biliary cystadenoma (IHBCA) was published in 1887 and the first resection was performed in 1892[1]. The tumor was redefined by Edmondson in 1958 as a multilocular lesion with an ovarian-like stroma[2]. However, in subsequent years, unilocular cystadenomas as well as cystadenomas without an ovarian-like stroma have been reported. Only 38 cases could be included in an extensive review in 1977[3]. With

the widespread availability of modern imaging techniques and developments in safe liver surgery, the number of reported cases increased to approximately 150 by 1994 (approximately 100 patients in the 1994 review by Devaney *et al*[2], and other earlier papers[4-6] not included in the review).

Biliary cystadenocarcinoma was first described in 1943[7]; a review published in 1998 included 113 patients[8]. Devaney *et al*[2] proposed three subsets of cystadenocarcinoma based on the pathology material submitted to their institutional laboratories for primary diagnosis or consultation: (1) cystadenocarcinoma originating from a benign cystadenoma with ovarian-like stroma (occurs exclusively in women); (2) *de novo* cystadenocarcinoma occurring almost only in men; and (3) cystadenocarcinoma that occurs in women but does not contain an ovarian-like stroma.

The long list of possibilities in the differential diagnosis includes simple cysts, parasitic cysts, degenerated metastatic tumors, mucin-producing metastatic tumors, congenital cystic dilation, cystic hemangioma, lymphangioma, hepatic foregut cyst, mesenchymal hamartoma and teratoma[2,9-11]. Imaging techniques are the primary diagnostic tools. However, the relative scarcity of the cystadenomas and cystadenocarcinomas diagnosed by different techniques and reported over a longer period than a century renders making definite statements on pathognomonic findings difficult. Also, the high frequency of simple cysts in patients older than 40 years of age (14%-24% depending on age) greatly complicates the problem in patients with unilocular cystadenomas[12]. It is possible that some IHBCAs are misdiagnosed as simple liver cysts.

IHBCA is a premalignant lesion; intrahepatic biliary cystadenocarcinoma (IHBCAC) cannot be reliably differentiated from IHBCA by imaging or preoperative aspiration cytology. Therefore both types of lesion should be excised[4,10,11,13-17].

In this article, we communicate our institutional experience on IHBCA and IHBCAC and review the related surgical literature.

MATERIALS AND METHODS

The charts of patients examined for cystic liver lesions between 2003 and 2008 were studied retrospectively.

The diagnosis of IHBCA was made by radiologic criteria (ultrasonography with computed tomography or magnetic resonance imaging). Important radiologic features were[18-23]: (1) Presence of a multilocular or unilocular mass with a well-defined capsule; and (2) Presence of one or more of the following structures exhibiting contrast enhancement: papillary projections, internal septations with nodular areas, wall thickness irregularities and mural nodules.

Because the necessity and utility of performing cyst fluid aspiration for tumor marker [carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9)] measurements and cytologic examination have been controversial issues until recently, the decisions in individual patients were left to the discretion of the attending surgeon.

Surgical intervention was performed if radiologic findings suggested an IHBCA or IHBCAC. All specimens were sent for histopathological examination. Frozen section was performed after enucleation procedures without any diagnosis of malignancy.

All patients were followed by computed tomography for possible recurrences every 6 mo in the first two postoperative years and then annually.

RESULTS

In the study period, 210 patients with cystic liver lesions were examined at our unit; 168 patients had parasitic cysts; 33 of the 42 nonparasitic cystic lesions were simple hepatic cysts. The final diagnoses in the remaining patients were IHBCA (n = 8) and IHBCAC (n = 1). These nine patients were all female with a median (range) age of 49 (27-60) years.

The most common symptom was abdominal pain observed in 6 patients. Three patients were asymptomatic; cystic liver masses had been discovered incidentally during radiological examinations for other purposes.

Four patients had undergone previous laparotomy (with other diagnoses) which resulted in incomplete surgery or recurrences. Two of these patients were operated on with the preoperative diagnosis of cystic echinococcal disease (one at our hospital). At surgery, the cystic lesions were misdiagnosed as simple liver cysts and unroofing was performed. However, histopathologic examination showed IHBCA in one and IHBCAC in the other. The remnant tumors in both patients were resected with appropriate surgical margins. Another patient was operated on with the diagnosis of echinococcal cyst at another hospital in the third month of her pregnancy. Operative findings did not confirm the preoperative diagnosis; a partial resection was performed and the histopathological diagnosis was IHBCA. In the course of the pregnancy, the size of the remnant cystic lesion increased from 12 to 27 cm in diameter. After a successful delivery, she was referred to our institution for hepatic surgery. A 58 year-old woman was operated on for cholecystolithiasis at another hospital; however, there was a suspicion of a malignant cystic lesion in segment V of the liver, the operation was stopped and she was referred to our hospital.

None of the patients had clinical or biochemical findings of cholestasis. Serum CEA levels were within normal range in all patients; serum CA 19-9 levels were within the normal range in 7 patients (including the single patient with IHBCAC) and were increased in 2 other patients (99 and 77 U/mL respectively; range 0-34 U/mL).

Preoperative percutaneous cyst fluid aspiration was performed in 4 patients. CA 19-9 levels were markedly increased in all samples (above 10000 U/mL; normal range for serum: 0-34 U/mL) and CEA levels were increased in 2 (15 and 18 ng/mL, respectively; normal range for serum: 0-4 ng/mL). Cyst fluid samples for postoperative examination were obtained intraoperatively in 4 other patients; both CA 19-9 (10000 U/mL and 379 U/mL) and CEA (27 U/mL and 651 U/mL respectively) were increased in 2 patients and within normal range in the other 2.

CEA and CA 19-9 measurement was not performed in the patient with cystadenocarcinoma.

Cytologic examination results were nondiagnostic, including the single patient with cystadenocarcinoma.

Preoperative evaluation of the period is the same as hepatobiliary operation's. The operative technique was determined according to the location of tumor in the liver and proximity to major vascular structures. Six patients were treated by hepatic resection: 4 by major hepatectomies (1 by right hepatic lobectomy, 1 by left hepatectomy, 1 by left lateral sectionectomy, and 1 by central bisegmentectomy) and 2 by nonanatomic resections. In 3 patients, the tumor was removed by enucleation. Enucleation was performed as in hemangiomas as described by Alper *et al*[24]. Frozen section was performed routinely after enucleation procedure and no invasive malignancy was diagnosed in these 3 patients. Therefore, no additional hepatic resection was performed.

Perioperative findings, length of the operation time and blood loss were uneventful in 9 patients.

There was no major complication and mortality.

Histopathologic examination revealed IHBCA in 8 patients and IHBCAC in 1. An ovarian-like mesenchymal stroma was observed in 8 patients including the patient with IHBCAC.

All patients were followed up for median (range) 31 (7-72) mo without recurrence.

DISCUSSION

Although the incidence of IHBCA and IHBCAC has been reported to be less than 5% of all hepatic cystic lesions[25], this figure, which is quoted in other papers[11,26] should be interpreted with caution since the frequency of simple cysts in patients older than 40 years of age varies between 14% and 24%[12]. The true incidences of both lesions are probably much lower since the largest surgical series reported includes 34 IHBCAs[13] and 6 IHBCACs[6]. The controversy in the literature stems from the lack of established criteria for preoperative diagnosis especially in the case of unilocular IHBCAs[4,10,27].

In spite of the improvements in imaging techniques, the differential diagnosis of simple hepatic cysts and IHBCAs is still problematic. In a Cleveland Clinic series, 10 of 18 patients underwent incorrect and unnecessary procedures such as percutaneous aspiration, ethanol injection, unroofing and omentoplasty[16]. In 1 of the patients in the present series, a patient with right upper abdominal quadrant pain was diagnosed as having cholecystolithiasis and a simple hepatic cyst in segment V of the liver. However, during surgery, the surgeon suspected the possibility of a cystic tumor and terminated the operation. Although radiologic features such as papillary projections, internal septations with nodular areas, wall thickness irregularities and mural nodules suggest the possibility of a IHBCA[28,29], all of these except papillary projections may be observed in simple cysts as well albeit at a lower frequency[29].

Liver echinococcal cysts pose another diagnostic problem in endemic countries[30]. In our series, 3 cases underwent inappropriate initial procedures with the misdiagnosis of hydatid disease. Although that absence of a germinative membrane and daughter cysts may have alerted the surgeons intraoperatively, their lack of experience precluded further interventions in the first operation. In 1 of these patients, the incidental observation of the natural history of an IHBCA under the hormonal milieu of pregnancy is interesting. The patient underwent unroofing of a 12 cm cyst at the 3rd month of pregnancy; the lesion size increased to 27 cm in a matter of 6 mo. This is in accordance with the female hormone-dependency of these lesions, previous observations in pregnant patients[11,25,31-34] and possible association with oral contraceptive use[34].

Although serum levels of CA19-9 and CEA may be increased in some patients[26,27,35-37], this is not a universal finding[10]. In the present series serum CA 19-9 levels were high in 2 patients (the single patient with IHBCAC not among them); all serum CEA levels were within the normal range.

Levels of cystic fluid CA 19-9 have been proposed "as a diagnostic help in liver cysts of unknown nature"[38] and some centers incorporated cyst fluid tumor marker (CA19-9 and CEA) measurements into their management algorithm[13]. However, definite diagnostic criteria for CA19-9 and CEA levels have not been established because the published data were largely limited to the reports on increased levels in small numbers of IHBCA patients without statistically robust comparison with levels in simple cysts. Consequently, the same problem occurred in the differentiation of IHBCAs and IHBCACs[26,35,36,38-40].

In the widely cited important contribution by Koffron *et al*[13], the cyst fluid CEA and CA 19-9 levels of 22 IHBCA patients were compared with the levels in 4 patients with simple cysts and 4 patients with polycystic liver disease. All 8 control cases had normal levels; in contrast CA19-9 was markedly increased in all IHBCA patients; there were mild to marked increases in CEA levels as well[13]. This paper was given serious consideration by some of our attending physicians who experienced dilemmas in some patients. For example, a 75-year-old woman underwent complete aspiration of two hepatic cysts in the right lobe; the CA 19-9 levels were above 10 000 U/mL whereas CEA levels were within the normal range. The presumptive diagnosis at that time was an IHBCA; surgery was not offered due to the comorbid illnesses. That she has not had a recurrence for 2.5 years suggests that the lesions might be simple cysts rather than cystadenomas and an operation would have been unnecessary.

Two important papers published in 2009 shed more light to this issue. Waanders *et al*[41] conducted cyst fluid CA 19-9 measurements in 109 polycystic liver disease patients and 24 simple cyst patients and detected "extremely high" levels in both groups. Although the absence of pathologic confirmation is a potential weakness in interpretation (i.e. some of the patients may have had unilocular cystadenomas), the universally increased levels in all 24 patients are strong evidence for increased levels in simple cysts. Although the number of patients in the other paper[29] is smaller (14 patients with hepatic simple cysts), a major

strength is that all patients had pathologically confirmed diagnoses. Both normal and dramatically increased CA 19-9 and CEA levels were detected in simple cyst patients; there were no significant differences between the simple cyst patients (n = 14) and IHBCA patients (n = 17). These recent data suggest that cyst fluid tumor marker levels do not provide additional information in patients with suspected IHBCA.

Cyst fluid cytology has not been found to be useful in the differentiation of IHBCA and IHBCAC[33,42] because demonstration of malignant cells is rare, i.e. a negative cytology result will give a false sense of security. Needle biopsy of papillary projections or mural nodules may be more useful for this purpose[13]; however this is generally unnecessary since there is a surgical indication for IHBCA and definite preoperative diagnosis of IHBCAC is not strictly required[8]. Since there is a risk of tumor cell implantation due to the aspiration procedure[42], routine aspiration of hepatic cystic lesions should be avoided.

There is a general consensus that an IHBCA should be removed completely either by enucleation or liver resection because lesser procedures are associated with recurrence rates as high as 90%[4,40,43,44]. Satisfactory results with enucleation using the dissection plane between tumor and liver tissue have been reported[4,13,45]. Enucleation, which allows maximum preservation of hepatic parenchyma, is an appropriate procedure for benign lesions. One concern is that the IHBCA may harbor a malignancy which may be missed by preoperative imaging. In such instances, enucleation would be inappropriate even in patients with noninvasive carcinoma[2]; therefore hepatectomy with negative surgical margins is preferred. Although frozen section examination may sometimes yield a false-negative result for cancer[42], it is still wise to perform it on samples from solid parts of enucleated tumors[13] because resection of the adjacent parenchyma may be conducted in patients with carcinoma. Some groups advocate routine resection for these lesions[27]. Left hepatectomy was performed in this series for the only patient with IHBCAC in whom the tumor was located at median and lateral sections. Two patients with lesions in lateral and 1 patient in posterior sections were treated by enucleation. Frozen section was performed after enucleations and no invasive malignancy was detected. Major hepatectomies had to be performed in 3 IHBCA patients with lesions very close to vascular structures. Nonanatomic resections were carried out in 2 cases.

In conclusion, with the improvement and widespread availability of radiologic modalities, cystic biliary liver neoplasms are being detected more frequently. However, the differential diagnosis from simple cysts and in endemic countries, from ecchinococcal cysts, is still challenging. Although there are no pathognomonic findings except for papillary projections (not present in many cases), radiological imaging finding such solid parts, papillary projections and septation or mural nodules in cystic lesion are the basis of preoperative diagnosis. Cyst fluid examination with cytology and CEA and CA 19-9 level measurement do not provide additional information. Partial resections are inappropriate. The treatment of choice is total excision either enucleation of IHBCAs and formal resection for IHBCACs and suspicious lesions.

COMMENTS

Background

Biliary cystadenomas and cystadenocarcinomas are both rare neoplasms of the biliary system. They may be easily misdiagnosed and operated on as simple cysts or hydatid cysts. Inappropriate drainage and unroofing operations result in recurrences. Reliable preoperative differentiation of the premalignant form-cystadenoma- and the malignant form cystadenocarcinoma is difficult except in obviously invasive lesions.

Research frontiers

Contrary to the previous popular opinion, recent data suggest that cyst fluid tumor marker levels do not provide additional information in patients with suspected intrahepatic biliary cystadenoma. Also, cyst fluid cytology has not been found to be useful in the differentiation of intrahepatic biliary cystadenoma and intrahepatic biliary cystadenocarcinoma, because demonstration of malignant cells is rare, i.e. a negative cytology result will give a false sense of security. Reliable techniques should be developed for reliable preoperative differential diagnosis of simple hepatic cysts, biliary cystadenomas and cystadenocarcinomas.

Innovations and breakthroughs

Surgical removal of the whole cyst with negative resection margins is recommended by many authors in order to avoid recurrences. In some cases, this is impossible because of the proximity to the vascular structures and importantly, aggressive surgery is unnecessary for a benign lesion. In three patients, the authors performed enucleation due to proximity to the vascular structures; frozen section revealed no malignancy. These patients have experienced no recurrence.

Applications

The treatment of choice is total excision; either enucleation of intrahepatic biliary cystadenomas and formal resection for intrahepatic biliary cystadenocarcinomas and suspicious lesions. Frozen section should be routine after enucleation.

Peer review

It's an interesting review of a very rare neoplasm of the biliary system.

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BRIEF ARTICLE

Analysis of the delayed approach to the management of infected pancreatic necrosis

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Abstract

AIM: To analyze outcomes of delayed single-stage necrosectomy after early conservative management of patients with infected pancreatic necrosis (IPN) associated with severe acute pancreatitis (SAP).

METHODS: Between January 1998 and December 2009, data from patients with SAP who developed IPN and were managed by pancreatic necrosectomy were analyzed.

RESULTS: Fifty-nine of 61 pancreatic necrosectomies were performed by open surgery and 2 laparoscopically. In 55 patients, single-stage necrosectomy could be performed (90.2%). Patients underwent surgery at a median of 29 d (range 13-46 d) after diagnosis of acute pancreatitis. Sepsis and multiple organ failure accounted for the 9.8% mortality rate. Pancreatic fistulae (50.8%) predominantly accounted for the morbidity. The median hospital stay was 23 d, and the median interval for return to regular activities was 110 d.

CONCLUSION: This series supports the concept of delayed single-stage open pancreatic necrosectomy for IPN. Advances in critical care, antibiotics and interventional radiology have played complementary role in improving the outcomes.

Key words: Necrosectomy; Infected necrosis; Pancreas; Severe acute pancreatitis; Inflammation

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INTRODUCTION

Severe acute pancreatitis (SAP) is a disease with high morbidity and mortality[1,2]. In the absence of specific effective therapy, management revolves around supportive care[3,4].

While the overall reported mortality of acute pancreatitis (AP) varies between 5% and 12%[2,5], SAP, which comprises around 10%-20% of AP, continues to have a high mortality rate of around 25%[6,7] due to organ failure and sepsis arising from infected pancreatic necrosis (IPN).

Management of IPN has been widely studied over the last few decades[6,8-20]. Data on indications, timing and technique of debridement for IPN are varied. However, while recent reports reflect the common theme of delayed surgery in IPN[21], the ideal debridement technique continues to be debated[6,12,15].

Reports regarding minimally invasive surgery for IPN are now being published[22-25]. Thus, if we are to develop

evidence-based guidelines for the management of IPN, rather than comparing outcomes with the relatively higher mortality encountered in some reports published a few decades ago, a more balanced comparison should particularly include results from larger series, and include some of the more recent series in which surgery for IPN has been complemented by advances in critical care, interventional radiology and broader spectrum antibiotics.

We have been performing open necrosectomy in a uniform manner for the last 10 years with conventional abdominal drainage without post-operative peritoneal lavage.

The purpose of our study was to analyze the feasibility and outcome of performing open necrosectomy for IPN in a delayed fashion.

MATERIALS AND METHODS

During the time period between January 1998 and December 2009, patients with SAP who developed IPN and were referred to the authors' center for surgical management were analyzed for this report.

At admission, patients were scored for severity, based on the APACHE Ⅱ scoring system[1,26], and were managed with resuscitation and intensive (supportive) care strategies. SAP was defined clinically by the presence of associated organ failure and/or local complications such as necrosis, abscess, or pseudocyst[27]. In addition, the patients were also defined as having SAP if the APACHE Ⅱ score was ⩾ 9.

At admission, all the patients classified as having SAP were admitted to the intensive care unit (ICU) where resuscitation was commenced. The patients were started on antibiotics, which were usually fluoroquinolones and metronidazole during the initial few years. However, the choice of antibiotic was changed to carbapenems (meropenem 500 mg 6 hourly, for 7 to 14 d) thereafter, owing to the sensitivity of the local microbiological flora and based on the reports of the ability of carbapenems to penetrate the necrosum[28]. Antifungals were commenced if the duration of antimicrobial therapy went beyond 7 d.

In patients with SAP, contrast-enhanced computed tomography (CECT) scan was performed for assessing the local severity by the Balthazar computed tomography (CT) severity index[29]. A CT severity index of ⩾ 7 was considered indicative of SAP. Nutrition was maintained by nasojejunal intubation and feeding. Percutaneous interventions were performed when clinically indicated, particularly in unstable patients as a temporizing measure or a bridge to surgery. The aim was to try and delay any intervention beyond the first 21 d.

Indications for surgery[23,30,31]: (1) Sepsis syndrome - clinical deterioration that is progressive with or without organ system failure and accompanied by fever and leucocytosis; (2) IPN - confirmed by fine needle aspiration (FNA) cytology and microbiological examination; (3) CECT showing extensive pancreatic necrosis with air pockets diagnostic of IPN; and (4) "Persisting unwellness" - in the form of abdominal pain, malaise, inability to tolerate a diet, general lack of well being, and continuing weight loss.

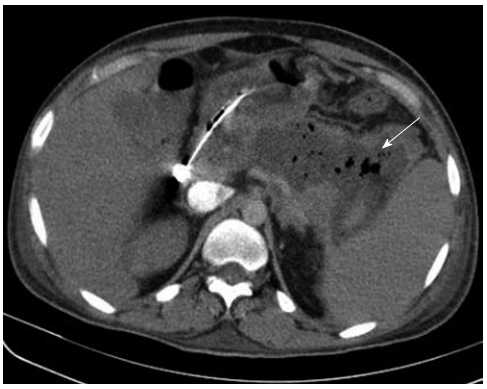

Figure 1 Contrast-enhanced computed tomography of the abdomen showing a large hypodense collection with air pockets in the location of the pancreatic body and tail (white arrow) indicative of an infected pancreatic necrosis.

The necrosectomy was planned as a single stage. Surgery was defined as delayed if it was performed at least 21 d after the onset of pain, which was considered as Day 0 of the attack. Fresh imaging in the form of CECT was obtained just prior to the exploration. The areas of necrosis and fluid collection were carefully mapped. The patient underwent a laparotomy through a transverse upper abdominal incision. Free fluid was aspirated, and the lesser sac was exposed either through the transgastrocolic or transmesocolic route. All the pus and fluid were removed and sent for microbiological examination. The necrotic debris was also removed carefully with blunt finger dissection and sponge-holders, with an attempt not to damage any of the normal tissue. Particular care was taken not to divide bands across the cavity, especially in areas where known vessels could cross, e.g. middle colic artery. Copious lavage with warm normal saline was performed, which also helped to separate the necrotic tissue from the normal tissue. Bleeding was controlled with temporary packing, after which specific vessels were underrun with non-absorbable sutures. Other areas were explored, depending on the CT interpretation. These included the right and left paracolic gutters, head of the pancreas, gastrohepatic omentum, pelvis, small bowel mesentery and the splenic hilum. Two 28 Fr tube drains were placed in the lesser sac and necrotic cavity. Loop ileostomy was performed selectively in the presence of extensive pericolic necrosis. Figure 1 is an abdominal CECT showing IPN. Figure 2 shows necrotic pancreas post-necrosectomy.

Post-operatively the patients were managed in the ICU. There was no attempt to perform post-operative lavage or flushing of the drains and the drains were removed once the output became minimal.

RESULTS

The 61 patients who required a necrosectomy for IPN included 49 male and 12 female patients. The mean age was 43 years (range 18-73 years). The predominant etiology for AP was gallstones (25 patients). Other etiologies included alcohol-induced (14 patients), idiopathic (13 pa-

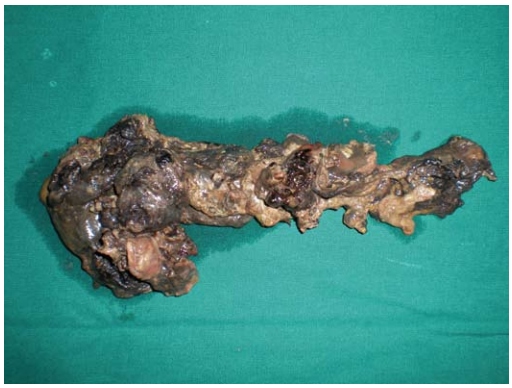

Figure 2 Post-operative photograph demonstrating a complete necrotic pancreas.

tients), traumatic (3 patients), post-endoscopic retrograde cholangio-pancreatography (3 patients), and metabolic (3 patients).

The median time of patient transfer to our institute, which is a tertiary care center, was 9 d (range 4-40 d). No patient had surgical intervention prior to transfer, but 4 patients had already undergone percutaneous (n = 2) or endoscopic (n = 2) drainage for fluid collections prior to transfer.

Fifty-nine patients underwent an open necrosectomy while 2 patients had a laparoscopic necrosectomy. Patients underwent surgery at a median of 29 d (range 13-46 d) from the onset of symptoms. In only one patient, the necrosectomy had to be performed on day 13 for unresponsive multiple organ dysfunction syndrome (MODS). Delayed necrosectomy could be performed in the other 60 patients (98.3%). Re-exploration was required in five (5/59, 8%) patients for ongoing necrosis. In these patients further exploration was required on an average 2.4 occasions (range 2-3 occasions). The rate of re-exploration was 8%. Two patients required subsequent percutaneous drainage for residual intra-abdominal collections. Overall, a single-staged open necrosectomy was successful in 55 (90%) patients.

The microbiological cultures obtained from the necrotic tissue showed evidence of organism growth in 51 patients (83.6%). Mixed gram-positive and -negative organisms were encountered in 9 cases. Of the organisms isolated, 46 cultures were positive for gram-negative organisms, predominantly *E. coli*, *Klebsiella*, *Acinetobacter* and *Pseudomonas* and 11 grew gram-positive organisms. Fungi were isolated in 9 cases, all of which were in bacterial-positive cultures.

The various complications encountered have been listed in Table 1. The most common complication encountered was pancreatic fistula. Other complications were bowel fistulae, bleeding, recurrent sepsis, wound infection and secondary fungal infection.

The diagnosis of pancreatic fistula was based on amylase estimation of the drain fluid, which ranged from 9000 to 104000 U/mL. The drainage tube was maintained *in situ* and the patient was managed on an outpatient basis. Complete healing was achieved in 20 patients after an average

Table 1 Complications encountered in the 61 patients and their management

Complication	*n* (%)	Management
Pancreatic fistula	31 (50.8)	Tube drainage-20
		Stenting-11
		Fistulojejunostomy-1
		Distal pancreatectomy-1
Enteric fistula	11 (18.0)	
Small bowel	2 (3.2)	Tube drainage-2
Large bowel	9 (14.7)	Defunctioning ileostomy-9
		Spontaneous healing-5
		Segmental colectomy-4
Bleeding	4 (6.5)	
Pseudo-aneurysm	3 (5)	Angioembolization-3
DIC	1 (1.6)	Platelets, factor Ⅶ
Secondary fungal infection	9 (14.7)	Antifungals
Wound infection	18 (29.5)	Wound drainage and dressings
Intestinal obstruction	3 (5)	Conservative-2
		Laparotomy-1
Pseudocyst	2 (3.2)	Cystojejunostomy-1
		Open drainage-1
Pelvic abscess	1 (1.6)	Pig tail drainage-1

DIC: Disseminated intravascular coagulation.

of 2 mo. Endoscopic stenting of the pancreatic duct was performed in 11 patients in whom the leak persisted for > 2 mo. Two patients required re-surgery in the form of a fistulojejunostomy and a distal pancreatectomy, as stent placement could not be achieved across the leak. Of the 11 patients who developed enteric fistulae in the post-operative period, 4 had undergone prophylactic ileostomy creation during the primary surgery due to the presence of extensive pericolic necrosis. Five patients required a loop ileostomy later. In 4 patients the colonic fistula healed without any sequelae, while 5 patients required segmental colectomy for colonic stricture or persistent leak for more than 6 mo. One patient died after colonic resection due to sepsis. The two patients with small bowel fistulae were managed conservatively with tube drainage. In 3 of the 4 patients who had post-operative hemorrhage, the source could be localized on angiography to pseudoaneurysms (splenic artery: 2, middle colic artery: 1) and this was managed by angioembolization. Another patient died due to coagulopathy and acidosis. Fifteen patients (24.5%) required readmission. The reasons for readmission included persistent pancreatic fistula (5 patients), colonic stricture (4 patients), intestinal obstruction (3 patients), pseudocyst formation (2 patients), and pelvic abscess formation (1 patient).

There were 6 deaths in the perioperative period with a mortality rate of 10%. These included 3 (of the five) patients who underwent re-explorations; 1 patient with post-operative hemorrhage, 1 patient with a colonic fistula and the patient who required an early necrosectomy. The cause of death was sepsis and MODS in all cases except the patient with hemorrhage. The median post-operative ICU stay was 7 d (range 3-30 d) and the median duration for which the patient required ventilatory support was 3 d (range 2-7 d). The median duration of hospital stay

following surgery was 23 d (range 11-88 d). The time to return to daily activity (defined as ability to perform daily personal activities, including feeding oneself and combing hair) was 16 d (10-20 d). The average time to return to regular activity was 110 d (60-140 d).

DISCUSSION

The ideal timing for a necrosectomy for IPN is a matter of debate. In our patients, we carried out a conservative management regimen with supportive care, antibiotics, early enteral feeding, and care of the patient in the ICU.

Using this management strategy we were able to perform a delayed necrosectomy, i.e. after 21 d, with potential benefits as follows*:* (1) Separation of viable from non-viable tissues making the operation technically easier; (2) Operating on a more hemodynamically stable patient; (3) Reduced bleeding as only non-viable tissue is removed[12]; (4) Removal of less normal pancreas resulting in reduced long-term morbidity[9]; and (5) Reduced local complications such as erosion into blood vessels/small bowel that could lead to post-operative hemorrhage or fistulae.

Mier *et al*[15] had previously put forward this principle of delayed surgery for IPN. The success of the approach was subsequently confirmed by other studies[6,13,21].

With this strategy of delaying surgery, in our series necrosectomy was performed as a single stage in all but 6 patients. In patients where the initially severe clinical course improved and the patient developed signs of sepsis in the third week, CT scan was repeated to map the extent of necrosis. At this time the pancreatic and peripancreatic necrosis tended to be localized with a resolution of the changes during the acute attack, such as acute fluid collections, stranding of the mesentery, *etc.*

The mortality rate in our study following the performance of a delayed single-staged necrosectomy was 9.8%. This compares favorably with the mortality rate of 11%-38% reported for open, as well as minimally invasive, necrosectomy for IPN over the last few years[8,10-12,17,18,23,32,33]. The indication for intervention in our patients was not solely based on an FNA as has been described previously[12]. We feel that FNA plays a role in the early period after SAP where it helps to differentiate systemic inflammatory response syndrome (SIRS) from infection. However, since we did not operate on the patients in this period, we did not find the need to apply the use of FNA as routine. Our decision to intervene was based on clinical parameters that included features such as persistent "unwellness", persistent pain in the abdomen, leucocytosis, appearance of a new fever especially after the second week when SIRS would not be a cause of raised temperature and infection of the pancreatic necrosis would be the only likely possibility, and the CT scan appearance of pancreatic necrosis[23,30,31].

The concept of delayed surgery has definitely been facilitated by improvements in critical care, fluid resuscitation and organ support that have contributed to the fall in the early mortality associated with SAP[34,35]. These have contributed by targeting one of the most important determinants of poor outcome in SAP, i.e. the early development and persistence of organ dysfunction[36].

We have used carbapenems, in particular meropenem, based on the proven efficacy of the drug for prophylaxis in patients with SAP[37]. The rationale for using antibiotics was that mortality for IPN is higher than that for sterile necrosis and antibiotic usage decreases the risk of infection[38,39]. The use of antibiotics indiscriminately, however, can lead to a 12%-35%[40,41] risk of opportunistic fungal infections, e.g. *Candida albicans* and *Aspergillus fumigatus*[42], which further increase the mortality rate[43,44]. The accepted indications for antibiotics in AP are: newly developed sepsis or SIRS, failure of two or more organ systems, proven infection, or an increase in serum C reactive protein in combination with other evidence supporting the presence of infection, e.g. CT scan[45]. We isolated fungal cultures in only 9 patients, i.e. 15% of cases, which was quite similar to the findings of Grewe *et al*[46] who reported similar fungal superinfections after using a four-drug regimen for a mean of 23 d.

The incidence of enteric fistulae in our study (18%) was comparable to that reported by Howard *et al*[47]. The incidence of developing a colonic fistula is high in patients with pericolic spread of necrosis into the left paracolic gutter, as seen in our patients, and we strongly advocate the use of prophylactic loop ileostomy in these patients. These results, along with those for pancreatic fistulae (50.8%), however, fall within the range of studies reporting post-necrosectomy gastrointestinal and pancreatic fistula rates of 1%-43% and 3%-72%, respectively[47]**.** The use of minimally invasive surgery has also been associated with enteric fistulae. In their series of 5 patients who underwent minimally invasive retroperitoneal necrosectomy, Lakshmanan *et al*[24] reported a 40% pancreatic fistula rate, while Connor *et al*[23] reported a 17% pancreatic fistula rate in their 24 patients. These results support the idea that such complications could largely be dependent on the nature of the disease rather than the procedure employed to treat it (open *vs* laparoscopy).

In our study we found that as a result of delaying the procedure beyond the first 3 wk, we were able to perform only a single, but effective, exploration in the vast majority of patients. Our re-operation rate was 8.2%, unlike that reported in other studies (22%-79%) where semi-open and open techniques of debridement, as well as early surgery, was practised[8,17,48-50].

The ideal time for intervening, as well as the number of interventions, has been shown to play a significant role on the mortality rate[6,21], as was seen in our study. Previous studies have stressed the significance of delayed necrosectomy. However, the best time for intervention continues to be controversial, though most studies have set the ideal time to be after 2-3 wk[6,13,15,51-56].

The value of a single-staged procedure is that it helps to avoid the risk of bleeding and fistula formation, as seen in patients undergoing open packing or re-operations[11,50,57]. The incidence of systemic complications is greater in patients who undergo re-operations[49].

Finally, benefit was also seen when we compared the duration of hospital stay with other studies (23 d *vs* 30-93 d)[8,17,23,32,40,49].

Our series provides further evidence to support the role of delayed open necrosectomy for IPN. The results are comparable, if not better, than reported smaller series using minimally invasive techniques. The results indicate that a multi-pronged conservative strategy aimed at supporting the patient, with timely intervention, may actually reduce the need for further interventions, reducing not only morbidity but also mortality in these patients.

In conclusion, this series provides further support to the concept of delayed single-stage open pancreatic necrosectomy for IPN. Advances in critical care, effective antibiotic therapy with carbapenems, the availability of interventional radiology and good supportive care have played a complementary role to surgery in improving outcomes in IPN. Prophylactic ileostomy may be considered in patients with necrosis extending into the paracolic gutters.

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COMMENTS

Background

Infected pancreatic necrosis (IPN) continues to have a high morbidity and mortality. Delayed necrosectomy has been shown to have reduced mortality. This paper illustrates a method of delayed necrosectomy performed in a single stage.

Research frontiers

The ideal technique of necrosectomy continues to be debated (open, endoscopic, laparoscopic). Reports regarding minimally invasive surgery for IPN are now being published. Thus, if we are to develop evidence-based guidelines for the management of IPN, rather than comparing outcomes with the relatively higher mortality encountered in some reports published a few decades ago, a more balanced comparison should particularly include results from larger series, and include some of the more recent series in which surgery for IPN has been complemented by advances in critical care, interventional radiology and broader spectrum antibiotics.

Innovations and breakthroughs

Previously described methods of necrosectomy have employed open packing, closed packing or post-operative lavage, which have high morbidity, cost and prolonged hospital stay. This single-stage delayed necrosectomy attempts to treat the patients in a single-stage approach, helping to reduce the hospital stay and morbidity.

Applications

This procedure helps to treat patients who have IPN with a high success rate, in addition to acceptable morbidity and low mortality. As it is carried out in delayed fashion, the necrotic debris has separated out and is well organized, leading to less intraoperative bleeding. This helps to pave the way for minimally invasive methods to further improve results.

Peer review

This is a well conducted retrospective study in agreement with the standard surgical approach to necrotic pancreatitis.

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BRIEF ARTICLE

Liver function alterations after laparoscopy-assisted gastrectomy for gastric cancer and its clinical significance

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Abstract

AIM: To evaluate the factors associated with liver function alterations after laparoscopy-assisted gastrectomy (LAG) for gastric cancer.

METHODS: We collected the data of gastrectomy patients with gastric cancer and divided them into 2 groups: open gastrectomy (OG) and LAG. We also collected the data of patients with colon cancer to evaluate the effect of liver manipulations during surgery on liver function alterations. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, and alkaline phosphatase were measured on the preoperative day and postoperative day 1 (POD1), POD3, POD5, and POD7.

RESULTS: No changes in liver function were observed after the operation in patients with colon cancer (n = 121). However, in gastric cancer patients (n = 215), AST and ALT levels increased until POD5 compared to those in colon cancer patients and these findings were observed both in the LAG and OG without a significant difference except at POD1. The mean hepatic enzyme levels at POD1 in the LAG group were significantly higher than those in the OG group (P = 0.047 for AST and P = 0.039 for ALT). The factors associated with elevated ALT on POD1 in patients with gastric cancer were body mass index (P < 0.001), operation time (P < 0.001), intraoperative hepatic injury (P = 0.048), and ligation of an aberrant left hepatic artery (P = 0.052) but not type of operation (OG *vs* LAG, P = 0.094).

CONCLUSION: We conclude that the liver function alteration after LAG may have been caused by direct liver manipulation or aberrant hepatic artery ligation rather than the CO_2 pneumoperitoneum.

Key words: Gastric cancer; Liver function; Pneumoperitoneum; Laparoscopy-assisted gastrectomy

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INTRODUCTION

While laparoscopic surgery has some limitations, such as a longer operating time and a more difficult surgical procedure than open surgery, it also has several advantages, such as less postoperative pain, an earlier recovery, and esthetic merits. A major difference between laparoscopic and open surgery is that CO_2 is used to create a pneumo-

peritoneum during laparoscopic surgery, which may result in respiratory and hemodynamic changes[1]. According to many studies, a laparoscopic cholecystectomy, a standard surgical procedure for gallbladder disease, results in an alteration of postoperative liver function more frequently than open cholecystectomy, and the cause of such a liver function alteration is believed to be the pneumoperitoneum created by the CO_2[1-5]. Based on previous studies, laparoscopy-assisted gastrectomy (LAG) is expected to result in liver function alterations resulting from the CO_2 pneumoperitoneum; this occurs because the CO_2 exposure time is much longer in LAG because of the difficulty of the surgical technique and a longer operating time compared to laparoscopic cholecystectomy.

We investigated whether liver function alterations take place in patients who undergo LAG for gastric cancer and whether other factors affect liver function besides a CO_2 pneumoperitoneum. We also investigated the effect of the liver function alterations on the clinical results. To do this, the postoperative liver functions of patients who underwent open gastrectomy (OG) and of patients who underwent LAG were compared. In addition, the postoperative liver functions of patients who underwent open colectomy (OC) and of patients who underwent laparoscopy-assisted colectomy (LAC) for colon cancer were compared.

MATERIALS AND METHODS

Patients

We collected data from 237 consecutive patients who received a radical gastrectomy for gastric cancer at Soonchunhyang University Bucheon Hospital between January 2006 and December 2007. Of these 237 patients, 22 were excluded: 6 had hepatic cirrhosis, 5 had undergone hepatic resection simultaneously with gastrectomy for benign hepatic diseases or hepatic metastasis, 10 had received endoscopic treatment for biliary tract stones before the surgery or intraoperatively had undergone biliary system surgery, and one patient had intraoperatively received a biliary system iatrogenic injury. These patients were excluded because the surgical techniques may have affected their liver function during surgery. However, patients whose liver function was normal, including those who had hepatitis or fatty liver before the operation, and patients who underwent a cholecystectomy simultaneously, were not excluded.

Patients who underwent a colectomy without direct liver manipulation during the operation were the control group for the gastrectomy patients because we thought that traction or manipulation of the liver during the operation might affect postoperative liver function. Of the 133 patients who underwent a radical colectomy for colon cancer during the same period, those included in the study were selected according to the same exclusion criteria.

The patients were divided into 4 groups based on diagnosis and surgical technique: LAG group, OG group, LAC group, and OC group.

Methods

The patients' clinicopathologic characteristics, hepatic disease history, surgical method, surgical outcome, and liver function before and after surgery were retrospectively examined using their medical records. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (Bil), and alkaline phosphatase (ALP) were measured preoperatively and examined at postoperative day 1 (POD1), POD3, POD5, and POD7. The normal reference levels were AST 5-40 IU/L, ALT 0-40 IU/L, Bil 0.2-1.2 mg/dL, and ALP 35-115 IU/L. Patients who had hepatic enzyme levels higher than the normal reference level were defined as the "elevated" group, and patients with a normal reference level were defined as the 'normal' group.

Surgical methods

The operations were performed in the supine position for OG patients, in the reverse-Trendelenburg position for LAG patients, and in the Trendelenburg position for OC and LAC patients. For LAG and LAC, CO_2 was insufflated *via* a trocar to insert the laparoscope, and the pneumoperitoneal pressure was maintained at 12 mmHg or less. In patients undergoing LAG and OG, the range of the gastric resection or lymph node dissection depended on the tumor location or preoperative TNM stage; for gastric cancer invading the submucosal layer or deeper, a D2 lymph node dissection was performed according to the Japanese Gastric Cancer Guidelines. In the LAG and OG groups, to provide an operative field in the upper pancreatic border, the left lobe of the liver was lifted when the lesser curvature of the stomach was dissected. For the LAC and OC groups, the extent of colon resection was also determined based on tumor location and lesion stage before the surgery, and no difference was observed between the 2 groups.

Statistical analysis

All values are expressed as the mean ± SD. The χ^2-test, the independent *t*-test, and the paired *t*-test were conducted using SPSS software (version 15.0 for Windows; SPSS Inc., Chicago, IL, USA). *P* values < 0.05 were considered statistically significant.

RESULTS

Patient clinicopathologic characteristics

Two hundred and fifteen patients with gastric cancer participated in our study (124 patients in the OG group, 91 patients in the LAG group). The mean age of the patients was 58.6 years, with 164 males and 51 females. Of the patients with gastric cancer, 31 (14.4%) had underlying liver diseases: fatty liver in 15 and viral hepatitis in 16. A combined operation was performed in 46 cases (21.4%), most frequently a splenectomy, followed by a cholecystectomy, adrenalectomy, or distal pancreatectomy. Stage I was the most common TNM stage in both the OG and LAG groups (137 patients, 63.7%), but significantly more stage I patients were in the LAG group (92.3%) than in

Table 1 Patient characteristics *n* (%)

Variables	Stomach cancer (*n* = 215)			Colon cancer (*n* = 121)		
	LAG (*n* = 91)	OG (*n* = 124)	*P*	LAC (*n* = 43)	OC (*n* = 78)	*P*
Gender (M:F)	70:21	94:30	NS	22:21	43:35	NS
Age (yr, mean ± SD)	57.1 ± 13.2	59.7 ± 11.9	NS	58.9 ± 10.3	62.0 ± 13.5	NS
BMI (kg/m^2, mean ± SD)	23.8 ± 3.2	23.5 ± 3.4	NS	24.2 ± 3.6	24.7 ± 2.9	NS
Underlying liver disease			NS			NS
No	81 (89.0)	103 (83.1)		30 (69.8)	66 (84.6)	
Yes	10 (11.0)	21 (16.9)		13 (30.2)	12 (15.4)	
Fatty liver	6	9		11	8	
Hepatitis	4	12		2	4	
TNM stage			< 0.001			0.004
Ⅰ	84 (92.3)	53 (42.7)		18 (41.9)	15 (19.2)	
Ⅱ	4 (4.4)	22 (17.7)		8 (18.6)	35 (44.9)	
Ⅲ	3 (3.3)	30 (24.3)		17 (39.5)	28 (35.9)	
Ⅳ	0	19 (15.3)		0	0	
Operation time (min, mean ± SD)	230.1 ± 77.6	165.2 ± 52.1	< 0.001	252.5 ± 77.2	184.1 ± 62.4	< 0.001
Combined operation			0.004			NS
No	80 (87.9)	89 (71.8)		42 (97.7)	74 (94.9)	
Yes	11 (12.1)	35 (28.2)		1 (2.3)	4 (5.1)	
Anesthetic agent			NS			NS
Sevoflurane	21 (23.1)	45 (36.3)		15 (34.9)	18 (23.1)	
Desflurane	70 (76.9)	79 (63.7)		28 (65.1)	60 (76.9)	
Postoperative morbidity	8 (8.8)	16 (12.9)	NS	1 (2.3)	5 (6.4)	NS
Postoperative mortality	1	1		1	0	
Hospital stay (d, mean ± SD)	10.7 ± 16.0	14.9 ± 12.6	NS	12.7 ± 3.8	17.9 ± 12.3	0.007

LAG: Laparoscopy-assisted gastrectomy; OG: Open gastrectomy; LAC: Laparoscopy-assisted colectomy; OC: Open colectomy; BMI: Body mass index; NS: Not significant.

the OG group ($P < 0.001$). A combined operation was performed more frequently in the OG group than in the LAG group ($P = 0.004$), and the operating time was significantly longer in the LAG group (203 min) than in the OG group (165 min, $P < 0.001$). No significant differences between the 2 groups were observed for age, presence or absence of underlying liver disease, type of anesthetic agent used, or postoperative complications.

Among the patients with colon cancer, 121 were included in the study. Their mean age was 60.9 years, and they consisted of 65 males and 56 females. No differences in gender, age, presence or absence of an underlying liver disease, combined operation rate, anesthetic agent, or postoperative complications were observed between the LAC (43 patients) and the OC groups (78 patients), whereas significant differences were found between the 2 groups for operating time and hospital stay after the operation, operating time being significantly longer in the LAC group and length of stay significantly longer in the OC group (Table 1).

Morbidity and mortality after the operation

Postoperative complications occurred in 24 (11.2%) patients who underwent a gastrectomy: wound infection in 7, anastomotic leakage in 5, postoperative bleeding in 5, intra-abdominal fluid collection in 5, and other complications in 2. Six cases of complications (5%) occurred in patients who underwent a colectomy: postoperative ileus in 3, anastomotic leak in one, and wound infection in one.

Two patients who underwent a gastrectomy (one each in the OG and LAG groups) and one patient who underwent a colectomy (LAC group) died after the operation. Thus, the postoperative mortality rate was 0.9% and 0.8% in patients who underwent a gastrectomy and colectomy, respectively. The causes of death were postoperative pneumonia (2 patients in the gastrectomy group) and sepsis (one patient in the colectomy group); no patient died of postoperative hepatic failure.

Postoperative changes in liver function

The postoperative AST and ALT levels were significantly higher than the preoperative levels in both the OG and LAG groups until POD5 (Table 2).

The mean hepatic enzyme levels at POD1 in the LAG group were significantly higher than those in the OG group ($P = 0.047$ for AST and $P = 0.039$ for ALT). No changes in preoperative or postoperative Bil and ALP levels were observed in either group.

No significant changes in the pre- and postoperative AST, ALT, Bil, and ALP levels were observed among patients who underwent a colectomy, and the changes were not significantly different between the LAC and OC groups (Table 2).

Approximately 65% of the patients in the LAG group and 59.7% in the OG group showed increased levels of AST at POD1, and 25.3% in the LAG group and 27.4% in the OG group showed increased levels of AST at POD3 ($P > 0.05$). Similar to the results with AST, 69.2% of the patients in the LAG group and 58.1% in the OG group showed increased levels of ALT at POD1, and 39.6% in the LAG group and 33.9% in the OG group

Table 2 Preoperative and postoperative liver enzymes (mean ± SD)

Variables	Stomach cancer (n = 215)			Colon cancer (n = 121)		
	LAG (n = 91)	OG (n = 124)	P^1	LAC (n = 43)	OC (n = 78)	P^2
AST (IU/L)						
Preoperative	22.1 ± 7.6	21.8 ± 7.3	NS	20.8 ± 8.7	19.9 ± 6.3	NS
POD1	73.8 ± 67.2[a]	57.6 ± 45.9[a]	0.047	23.2 ± 11.8	22.2 ± 11.5	NS
POD3	41.1 ± 43.5[a]	38.4 ± 24.3[a]	NS	22.9 ± 9.5	22.4 ± 8.4	NS
POD5	28.9 ± 24.1[a]	29.5 ± 18.1[a]	NS	25.3 ± 10.8	22.3 ± 10.8	NS
POD7	24.0 ± 11.6	22.2 ± 11.3	NS	28.9 ± 26.2	24.7 ± 13.4	NS
ALT (IU/L)						
Preoperative	23.9 ± 13.5	23.8 ± 15.8	NS	24.1 ± 3.7	17.0 ± 2.6	0.045
POD1	89.1 ± 103.7[a]	63.3 ± 65.9[a]	0.039	18.4 ± 8.1	17.4 ± 15.0	NS
POD3	62.2 ± 97.3[a]	51.5 ± 57.6[a]	NS	16.6 ± 6.5	15.9 ± 9.6	NS
POD5	43.2 ± 49.1[a]	39.4 ± 40.6[a]	NS	20.6 ± 13.9	16.4 ± 8.5	NS
POD7	33.7 ± 25.4	26.9 ± 17.9	0.033	32.1 ± 43.8	21.0 ± 25.4	NS
Total bilirubin (mg/dL)						
Preoperative	0.6 ± 0.2	0.5 ± 0.3	NS	0.5 ± 0.2	0.5 ± 0.3	NS
POD1	0.8 ± 0.3	0.7 ± 0.3	NS	0.7 ± 0.4	0.6 ± 0.3	NS
POD3	0.8 ± 0.4	0.7 ± 0.5	NS	0.4 ± 0.2	0.4 ± 0.2	NS
POD5	0.7 ± 0.3	0.6 ± 0.3	NS	0.5 ± 0.2	0.4 ± 0.2	NS
POD7	0.7 ± 0.3	0.5 ± 0.2	NS	0.5 ± 0.2	0.4 ± 0.2	NS
ALP (IU/L)						
Preoperative	65.6 ± 17.6	65.8 ± 22.1	NS	69.1 ± 20.3	64.4 ± 22.2	NS
POD1	46.7 ± 11.9	47.8 ± 14.6	NS	49.9 ± 15.5	44.6 ± 13.3	NS
POD3	44.2 ± 10.5	45.8 ± 14.5	NS	44.1 ± 11.7	41.9 ± 10.2	NS
POD5	50.6 ± 23.5	48.6 ± 15.6	NS	47.5 ± 18.7	43.2 ± 11.3	NS
POD7	57.9 ± 38.3	54.7 ± 22.6	NS	51.1 ± 24.1	47.2 ± 13.1	NS

[1]P values represent the statistical difference between the laparoscopy-assisted gastrectomy (LAG) and open gastrectomy (OG) groups; [2]P values represent the statistical difference between the laparoscopy-assisted colectomy (LAC) and open colectomy (OC) groups; [a]$P < 0.05$ *vs* preoperative liver enzymes using the paired t-test. POD: Postoperative day; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; NS: Not significant.

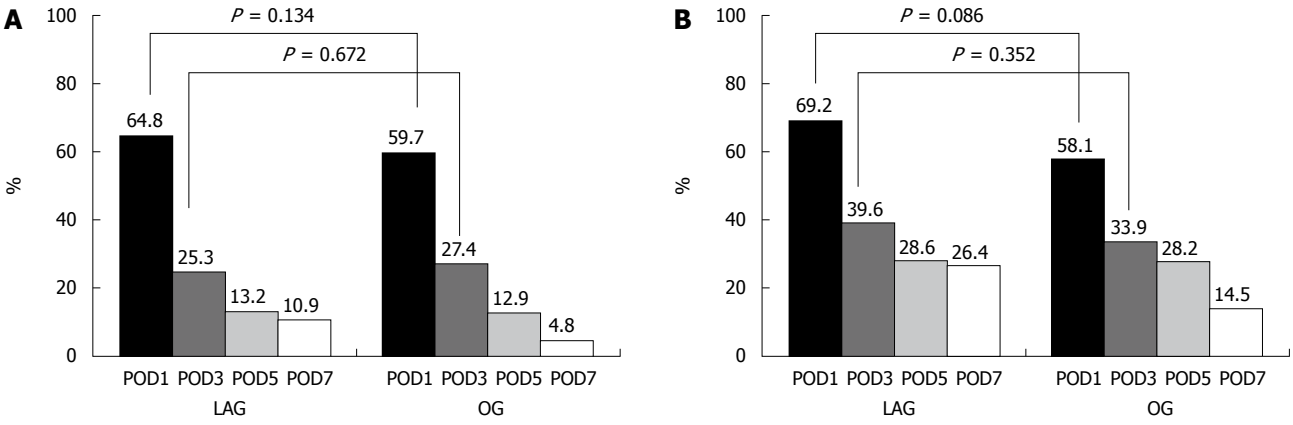

Figure 1 Frequency of patients who had elevated liver enzyme levels after a gastrectomy for gastric cancer. A: Aspartate aminotransferase; B: Alanine aminotransferase. LAG: Laparoscopy-assisted gastrectomy; OG: Open gastrectomy; POD: Postoperative day.

showed increased levels at POD3 ($P > 0.05$, Figure 1).

Among the patients who underwent a gastrectomy, 13 showed more than a 5-fold increase in ALT levels from the normal level at POD1 (8 patients in the LAG group and 5 patients in the OG group). Of these 13 patients, 3 showed more than a 10-fold increase (2 patients in the LAG group and one patient in the OG group).

Factors that affected postoperative liver function after gastrectomy

Unlike LAG, no change in liver function was observed after LAC when using a CO_2 pneumoperitoneum. Therefore, the characteristics of the patients who showed liver function alterations after LAG were examined to identify other factors that could cause changes in postoperative liver function. For this purpose, we investigated the ALT level at POD1, at which the change in postoperative liver function for those who underwent a gastrectomy was most prominent. The patients who underwent a gastrectomy were classified into 2 groups: a normal group that had a normal ALT range and an elevated group having higher ALT levels than the reference. We also examined their clinical features and surgical findings.

The changes in the ALT levels at POD1 for patients

Table 3 Comparison of alanine transaminase levels on the first postoperative day in patients who underwent a gastrectomy *n* (%)

Variables	Normal group (*n* = 80)	Elevated group (*n* = 135)	*P* value
Gender (M:F)	59:21	105:30	0.502
Age (yr, mean ± SD)	58.6 ± 13.4	58.6 ± 12.0	0.981
BMI (kg/m^2, mean ± SD)	22.3 ± 3.3	24.4 ± 3.0	< 0.001
Underlying liver disease			0.153
No	70 (87.5)	114 (84.4)	
Yes	10 (12.5)	21 (15.6)	
Fatty liver	2 (2.5)	13 (9.7)	
Hepatitis	8 (10.0)	8 (5.9)	
Operation time (min, mean ± SD)	168.9 ± 61.4	206.7 ± 73.6	< 0.001
Types of operation			0.094
Open	52 (65.0)	72 (53.3)	
Laparoscopy-assisted	28 (35.0)	63 (46.7)	
Combined operation			0.093
No	58 (72.5)	111 (82.2)	
Yes	22 (27.5)	24 (17.8)	
Anesthetic agent			0.979
Sevoflurane	24 (30.0)	40 (29.6)	
Desflurane	56 (70.0)	95 (70.4)	
Intraoperative transfusion			0.894
No	71 (88.8)	119 (88.1)	
Yes	9 (11.3)	16 (11.9)	
Aberrant left hepatic artery			0.052
Absence or preservation	79 (98.8)	127 (94.1)	
Artery ligation	1 (1.3)	8 (5.9)	
Intraoperative hepatic injury			0.048
No	79 (98.8)	125 (92.6)	
Yes	1 (1.3)	10 (7.4)	
Mean ALT at POD1 (± SD)	28.2 ± 8.1	101.4 ± 97.1	< 0.001

BMI: Body mass index; POD: Postoperative day; ALT: Alanine aminotransferase.

who underwent a gastrectomy were not significantly affected by gender, age, underlying liver disease, presence or absence of postoperative complications, type of anesthetic agent, or intraoperative transfusion. The body mass index (BMI) was significantly higher in the elevated group than in the normal group, and the operating time in the elevated group was significantly longer than in the normal group. Furthermore, 7.4% of the patients who experienced intraoperative hepatic injury showed increased ALT levels, which was a significantly higher proportion than in the patients who did not experience intraoperative hepatic injury (P = 0.048). Of the patients with normal ALT levels, 1.3% of patients had ligation of an aberrant left hepatic artery during the operation, whereas in the elevated group, 5.9% of patients had ligation of this artery, although this result had no statistical significance (P = 0.052). Thirty-five percent of the patients in the normal group underwent LAG, whereas 46.7% of the patients in the elevated group underwent LAG, but no significant difference was found (P = 0.094) (Table 3).

DISCUSSION

Pneumoperitoneum, which is created by CO_2 insufflation during a laparoscopic operation, results in postoperative transient liver function abnormalities. Halevy *et al*[6] first reported that aminotransferase levels increased significantly after laparoscopic cholecystectomy. They suggested that the increase in aminotransferase was due to increased intra-abdominal pressure, pressure against the liver with the lifting of the gall bladder, damage to the liver parenchyma by electrocautery, deformity of the extrahepatic biliary duct, the possibility of introducing a small stone into the biliary duct, or damage to the left hepatic artery during surgery. Other studies have reported that a laparoscopic cholecystectomy was more likely to result in liver function abnormalities than an open cholecystectomy and suggested that the cause of the abnormalities was reduced blood flow into the hepatic portal vein as a result of the increased intra-abdominal pressure caused by the pneumoperitoneum; the degree of pneumoperitoneal pressure is related to the degree of liver function abnormality[2,7].

Animal studies have also shown that a pneumoperitoneum directly damages rat liver tissue, that the degree of damage is related to pneumoperitoneal pressure, and that the persistence of the pressure at 15 mmHg or more for 60 min or longer may result in irreversible hepatic damage[8]. Contrary to these reports, a study on 1034 patients who underwent laparoscopic cholecystectomy reported that only 3.9% of the patients had a mild elevation in hepatic enzyme levels after the operation, and that the number of liver function abnormality cases after laparoscopic cholecystectomy was much lower than in other studies[9].

Very few studies have reported liver function abnormalities after LAG for gastric cancer. Etoh *et al*[10] and Kim *et al*[11] reported that liver function abnormalities occurred in patients who underwent LAG but not in those who had OG. They asserted that liver function abnormalities may occur after LAG due to the CO_2 pneumoperitoneum, although objective evidence for their assertion was unavailable.

Because LAG is more difficult to perform than OG, the operating time can be longer, and consequently, the exposure time of the intra-abdominal organs to the pneumoperitoneum may also be longer than in OG. Given this, a pneumoperitoneum was expected to cause liver function abnormalities. Thus, this study sought to determine whether postoperative liver function abnormalities would occur in patients who underwent LAG.

The change in the absolute hepatic enzyme levels was more marked in the LAG group than in the OG group. Moreover, consistent with the results of previous studies, this change was most prominent on POD1, but the level returned to normal on POD5. When the patients were divided into an elevated enzyme group and a normal group, the frequency of change in the absolute hepatic enzyme levels in the elevated group did not differ between the LAG and OG groups (Figure 1). Furthermore, when the patients who underwent a colectomy, in which the hepatic parenchyma and the vessels around the liver were not excessively manipulated, were divided into LAC and OC groups, no liver function abnormalities were found in either group. Thus, we presumed that the major cause of liver function abnormalities after LAG may not be the pneumoperitoneum. Similar to this result, Nguyen *et al*[12]

also reported no difference in the increase in hepatic enzyme levels between patients who underwent laparoscopic and open gastric bypass. They performed laparoscopic and open Roux-en-Y gastric bypass on obese patients and found that the hepatic enzyme level increase was highest in the first 24 h after the operation, and that liver function returned to normal 72 h after the operation. They also reported no significant difference in the hepatic enzyme level increase between those who underwent a laparoscopic *vs* an open operation, and they asserted that a prolonged pneumoperitoneum in patients who underwent the laparoscopic operation would not have greatly affected the change in the postoperative hepatic enzyme levels. The statistical significance of the effect of prolonged operating time on the increase in hepatic enzyme level after gastrectomy in the present study can be attributed to the long exposure time to the pneumoperitoneum. However, it can also be attributed to the long ischemic time of the liver due to extended liver traction.

Thus, we divided our patients into a group with normal liver function and a group with elevated liver function, based on the ALT level at POD1, at which the liver function abnormality was most prominent, to determine whether liver function after LAG is influenced by factors other than a pneumoperitoneum. The change in liver function was based on the ALT level because ALT exists mostly in the liver, unlike AST, and is the gold-standard clinical chemistry marker for liver injury[13,14]. The classification of the elevated group was based on the normal ALT reference range of 0-40 IU/L, which is used at the authors' hospital. As a result, the surgical technique (OG and LAG) did not affect the increase in the ALT levels after the operation, but the BMI in the elevated group was significantly higher than in the normal group. Also, the operating time was significantly longer in the elevated group than in the normal group. When the hepatic parenchyma was damaged or when the aberrant left hepatic artery was ligated, the ALT level increased.

Many studies on liver function abnormalities after a laparoscopic operation have reported that the cause of postoperative liver function abnormality was hepatic ischemia caused by reduced portal flow due to the pneumoperitoneum[8]. However, we did not find any liver function abnormalities after LAC, whereas liver function abnormalities after an open gastrectomy were found when the hepatic parenchyma was directly damaged or when the aberrant hepatic artery was ligated. Given these conditions, the causes of liver function abnormalities after LAG can be attributed to excessive liver traction, direct damage to the hepatic parenchyma, ligation of an aberrant hepatic artery during the operation, or reduced portal flow due to the pneumoperitoneum.

Patients who underwent a gastrectomy showed significantly increased hepatic enzyme levels on POD1, regardless of the surgical technique, which returned to normal on POD5. This transient liver function abnormality was not clinically meaningful because it did not cause hepatic failure or clinical symptoms. However, as patients with hepatic cirrhosis or decreased liver function were not included in this study, further research is required to determine whether LAG is safe for patients with decreased liver function. The assumption was made that LAG could be performed safely in patients with decreased liver function by taking the greatest possible care with ligation of the aberrant hepatic artery and not damaging the hepatic parenchyma, or if the major cause of the liver function abnormality after LAG was damage to the hepatic parenchyma due to excessive liver traction.

COMMENTS

Background

A major difference between laparoscopic and open surgery is that carbon dioxide (CO_2) is used to create a pneumoperitoneum during laparoscopic surgery, which may result in respiratory and hemodynamic changes. According to many studies, a laparoscopic cholecystectomy, a standard surgical procedure for gallbladder disease, results in an alteration of postoperative liver function more frequently than open cholecystectomy, and the cause of such liver function alteration is believed to be the pneumoperitoneum created by CO_2. Based on previous studies, laparoscopy-assisted gastrectomy (LAG) is expected to result in liver function alterations due to the CO_2 pneumoperitoneum.The paper investigated whether liver function alterations take place in patients who undergo LAG for gastric cancer and whether other factors affect liver function besides a CO_2 pneumoperitoneum.

Innovations and breakthroughs

In the study, patients who underwent a colectomy without direct liver manipulation during the operation were the control group for the gastrectomy patients because the authors thought that traction or manipulation of the liver during the operation might affect postoperative liver function. The change in the absolute hepatic enzyme levels was more remarkable in the LAG group than in the open gastrectomy (OG) group. Moreover, consistent with the results of previous studies, this change was most prominent on postoperative day 1 (POD1), but the level returned to normal on POD5. When the patients were divided into an elevated group and a normal group, the frequency of change in the absolute hepatic enzyme levels in the elevated group did not differ between the LAG and OG groups. Furthermore, when the patients who underwent a colectomy, in which the hepatic parenchyma and the vessels around the liver were not excessively manipulated, were divided into laparoscopy-assisted colectomy (LAC) and open colectomy groups, no liver function abnormalities were found in either group. Thus, authors presumed that the major cause of liver function abnormalities after LAG may not be the pneumoperitoneum.

Applications

Many studies on liver function abnormalities after a laparoscopic operation have reported that the cause of postoperative liver function abnormality was hepatic ischemia caused by reduced portal flow due to the pneumoperitoneum. However, authors did not find any liver function abnormalities after LAC, whereas liver function abnormalities after an open gastrectomy were found when the hepatic parenchyma was directly damaged or when the aberrant hepatic artery was ligated. Given these conditions, the causes of liver function abnormalities after LAG can be attributed to excessive liver traction, direct damage to the hepatic parenchyma, ligation of an aberrant hepatic artery during the operation, or reduced portal flow due to the pneumoperitoneum. The assumption was made that LAG could be performed safely on patients with decreased liver function by taking the greatest possible care with the aberrant hepatic artery ligation and not damaging the hepatic parenchyma, or doing so if the major cause of the liver function abnormality after LAG was damage to the hepatic parenchyma due to excessive liver traction.

Peer review

This is a well designed and researched paper. The positive findings show more marked changes in liver function after a laparoscopy-assisted gastrectomy for gastric cancer than after an open gastrectomy, lasting for up to 5 d, these differences are related to body mass index (BMI) and longer operation time. The authors could have suggested that these could be explained by the heavier retraction of the liver which would be required in patients with a greater BMI and the greater duration of the retraction in the laparoscopy group.

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BRIEF ARTICLE

Necrotic stercoral colitis: Importance of computed tomography findings

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Abstract

AIM: To study the computed tomography (CT) signs in facilitating early diagnosis of necrotic stercoral colitis (NSC).

METHODS: Ten patients with surgically and pathologically confirmed NSC were recruited from the Clinico-Pathologic-Radiologic conference at Chang Gung Memorial Hospital, Taoyuan, Taiwan. Their CT images and medical records were reviewed retrospectively to correlate CT findings with clinical presentation.

RESULTS: All these ten elderly patients with a mean age of 77.1 years presented with acute abdomen at our Emergency Room. Nine of them were with systemic medical disease and 8 with chronic constipation. Seven were with leukocytosis, two with low-grade fever, two with peritoneal sign, and three with hypotensive shock. Only one patient was with radiographic detected abnormal gas. Except the crux of fecal impaction, the frequency of the CT signs of NSC were, proximal colon dilatation (20%), colon wall thickening (60%), dense mucosa (62.5%), mucosal sloughing (10%), perfusion defect (70%), pericolonic stranding (80%), abnormal gas (50%) with pneumo-mesocolon (40%) in them, pericolonic abscess (20%). The most sensitive signs in decreasing order were pericolonic stranding, perfusion defect, dense mucosal, detecting about 80%, 70%, and 62.5% of the cases, respectively.

CONCLUSION: Awareness of NSC and familiarity with the CT diagnostic signs enable the differential diagnosis between NSC and benign stool impaction.

Key words: Fecal impaction; Dense mucosa; Pericolonic stranding; Stercoral colitis; Computed tomography

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INTRODUCTION

Necrotic stercoral colitis is a necrotic process that occurs

in stercoral colitis (SC), caused by fecal impaction that results in pressure ulceration and regional necrosis. Perforation is rare, but has a mortality rate of 32%-57%[1]. Early diagnosis with aggressive bowel cleansing and disimpaction may decrease the pressure and lessen the likelihood of ulceration of the colon[2]. Fecal impaction frequently occurs in elderly patients, and those who are bed-ridden for a prolonged period of time.

Most patients present to the emergency room (ER) with an acute abdomen. Their physical examinations and laboratory data are often unreliable. Moreover, the peritoneal signs are often nonspecific and might be attributed to diverticulitis, which is more common in elderly patients[3]. Computed tomography (CT) is readily available and is not operator-dependent; therefore, abdominal CT is often requested by emergency physicians to evaluate patients with acute abdominal conditions.

Very little has been published on NSC in the radiology literature[3]. We reviewed the CT findings of 10 patients with NSC from our hospital, to call attention to this potentially fatal condition.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by Chang Gung Memorial Hospital (98-0044B).

Patients

Between November 2002 and August 2009, ten patients with surgically and pathologically confirmed NSC were recruited from the Clinico-Pathologic-Radiologic conference at Chang Gung Memorial Hospital, Taoyuan, Taiwan. We reviewed their abdominal radiographs, CT images, and medical records retrospectively.

CT protocol

All of these patients underwent CT examinations of the abdomen and pelvis before surgical exploration, while they stayed in the ER. CT examinations were performed by four-detector CT (LightSpeed QX/i Scanner, General Electric Medical Systems, Milwaukee, WI, USA). Helical CT images were acquired using either 7- or 5-mm slice collimation, reconstruction interval of 5 mm, pitch of 1.5-2, 120 kV, and 200-240 mA. One hundred milliliters of intravenous (IV) contrast agent was used routinely.

CT interpretation

Several CT findings of fecal impaction in the colon, thickening of the colon wall, and pericolonic stranding indicated SC, whereas the presence of extraluminal gas bubbles or an abscess suggested that perforation had occurred[2].

The CT examinations were retrospectively reviewed by two independent board-certified abdominal radiologists who were blinded to the CT official reports and the surgical and pathologic findings. They viewed the CT images on a picture archiving and communication system (PACS) independently and discussed the findings until consensus was reached. If consensus could not be reached, a third abdominal radiologist was consulted. All abdominal radiographs were reviewed for abnormal gas. They were also requested to determine the presence or absence of the CT features of NSC, including location of fecal impaction, proximal colon dilatation, colon wall thickening, dense mucosa, mucosal sloughing, perfusion defect, pericolonic stranding, pericolonic abscess, and abnormal gas with or without pneumo-mesocolon. Vascular ischemic colitis was excluded based on patency of the inferior mesenteric artery and vein.

Definition of CT signs

The individual CT signs were defined as follows - Fecal impaction: distended colon with much feces or packing of dehydrated fecaloma in the colon; Proximal colon dilatation: a distended left-sided colon with a cylindrical shape and cross-sectional diameter > 6 cm; Colon wall thickening: regional wall thickness > 3 mm in the obstruction site; Dense mucosa: increased mucosal lining density on pre-contrast CT; Mucosal sloughing: mucosa dislodged into the lumen; Perfusion defect: discontinuity of the enhancement of colon mucosa or apparently decreased enhancement as compared with adjacent small bowel loops; Pericolonic stranding: increased streaks of pericolonic fat; Pericolonic abscess: pericolonic loculated fluid or mottled substance; Abnormal gas: gas migrating into or beyond the colon wall as pneumoperitoneum or pneumoretroperitoneum, i.e. pneumo-intestinalis coli: gas entrapped in the mural wall; pneumo-mesocolon: gas confined inside the mesocolon; and portal vein gas: air leakage into the portomesenteric vessels.

RESULTS

Demography and clinical information

Six men and four women aged 39-88 years (mean, 77.1 years) were studied (Table 1). All of the patients presented to our ER with acute abdomen. Chronic constipation and systemic medical disease were the common clinical problems in these patients. Abdominal discomfort was not greatly improved after local removal of impacted feces by digital evacuation or fleet enema. On arrival at the ER, two patients (20%) presented with a low grade fever (< 38.5℃), two (20%) presented with peritoneal signs, and seven (70%) presented with leukocytosis with one other at borderline criteria of leukocytosis. Three patients (30%) arrived at the ER with hypotensive shock (systemic blood pressure < 90 mmHg). Surgical intervention was indicated for all of the patients. Seven of the patients died; thus, the mortality rate was 70%. Among these seven patients, three died within 1 wk, highlighting the rapidly progressive course of the disease.

CT signs

The imaging findings of NSC are listed in Table 2. CT

Table 1 Clinical data of study patients with necrotizing stercoral colitis

No.	Age (yr)/sex	sBP	BT	Hx	Cor	PS	WBC	TI	Fe	Pe	Operation findings	Pathology	Outcome
1	76/M	183	38.4	+	DM, HTN Arrhythmia	-	22.2k/89	5′30″	RS	No	Ischemic change from sigmoid to rectum with necrotic mucosa	Ischemia necrosis with mucosal sloughing	Alive
2	86/M	130	34.4	+	CAD RF	-	45.6k/72	2′30″	S	S	Necrosis of descending and sigmoid colon with a 2-cm perforation	Perforating ulcer with transmural necrosis	Dead, 1 d after CT
3	79/F	147	36.1	+	DM	-	15.3k/76	7 d	D	D	Necrosis of nearly entire colon, with a 1.7-cm perforation	Mucosal ulcer with perforation	Dead, 19 d after CT
4	87/M	158	35.6	+	HTN	+	14.4k/90	4′40″	RS	S	A 2-cm perforator 2 cm proximal to the recto-sigmoid colon cancer	Transmural necrosis with a 2.1 cm perforator	Alive
5	80/F	120	33.6	+	HTN	-	3.6k/67	24′30″	S	S	Nearly entire colon necrosis with a 5-cm × 3-cm perforator at sigmoid colon	Ulcerative hole with transmural necrosis at sigmoid colon	Dead, 5 d after CT
6	70/F	145	36.4	+	DM	-	13.3k/80	15′40″	RS	No	Necrosis of distal ileum and entire colon	Transmural necrosis of bowel wall	Dead, 47 d after CT
7	88/F	81	35.0	+	-	-	4k/38	3′	RS	S	2 small perforators at proximal sigmoid colon	Gangrenous change with transmural necrosis of sigmoid colon	Dead, 8 d after CT
8	39/M	64	38.0	NA	ESRD	+	9.9k/79	26′	RS	No	Ischemic patches over sigmoid colon with impending perforation	Ischemic and gangrenous change of the sigmoid coon	Dead, 3 d after CT
9	83/M	158	37.0	NA	ARDS, HF HTN, COPD	-	17k/93	11′	RS	No	Ischemic change of small bowel and sigmoid colon	Transmural necrosis of sigmoid colon and mucosal necrosis of small bowel	Dead, 11 d after CT
10	83/M	64	35.3	+	CAD, HTN	-	46k/83	10′	RS	No	Patch necrosis of the T and D colon	Gangrenous change of the T and D colon	Alive

sBP: Systemic blood pressure (mmHg); BT: Body temperature (℃); Hx: History of constipation; Cor: Comorbidity; PS: Peritoneal signs at initial admission physical examination; WBC: White blood cell (number/percentage of segment) at admission; CT: Computed tomography; TI: Time interval between CT and surgery; Fe: Stool obstructive site; Pe: Perforator site; CAD: Coronary arterial disease; RF: Renal failure; HTN: Hypertension; HF: Heart failure; NA: Not applicable; -: Absent; +: Present; 5′30″: 5 h and 30 min, *etc*.; RS: Recto-sigmoid colon; S: Sigmoid colon; T: Transverse colon; D: Descending colon; DM: Diabetes mellitus; ESRD: End-stage renal disease; ARDS: Acute respiratory distress syndrome; COPD: Chronic obstructive pulmonary disease.

Table 2 Imaging signs of necrotizing stercoral colitis

No.	Radiographic abnormal gas	CT signs										
		Fecal impaction	Obstructive site	Proximal dilatation	Wall thickening	Dense mucosa	Mucosal sloughing	Perfusion defect	Pericolonic stranding	Abnormal gas	Pneumo-mesocolon	Abscess
1	N	Y	RS	N	N	NA	N	Y	Y	N	N	N
2	Y	Y	S	N	Y	NA	N	Y	N	Y	Y	N
3	N	Y	D	N	N	Y	N	N	Y	Y	N	N
4	N	Y	RS	N	Y	Y	N	Y	Y	Y	Y	Y
5	N	Y	S	N	Y	Y	N	Y	Y	Y	N	N
6	N	Y	RS	Y	Y	Y	N	Y	N	N	N	N
7	N	Y	RS	N	Y	Y	Y	Y	Y	Y	N	Y
8	N	Y	RS	Y	N	N	N	N	Y	N	N	N
9	N	Y	RS	N	Y	N	N	Y	Y	N	N	N
10	N	Y	RS	N	N	N	N	N	Y	N	N	N
Frequency	1/5 (20%)	10/10 (100%)	9/10 (90%)	2/10 (20%)	6/10 (60%)	5/8 (62.5%)	1/10 (10%)	7/10 (70%)	8/10 (80%)	5/10 (50%)	2/5 (40%)	2/10 (20%)
κ-value	1	1	1	1	0.4	0.714	0.615	0.286	0.737	0.8	1	1

CT: Computed tomography; NA: Not applicable; N: No; Y: Yes; RS: Recto-sigmoid colon; S: Sigmoid colon; D: Descending colon.

examination revealed fecal impaction at the sigmoid colon in nine patients (90%) and at the distal descending colon in one (10%). Proximal colon dilatation was found in two patients (20%). Colon wall thickening (Figure 1) occurred in six patients (60%), dense mucosa (Figure 2A) in five (62.5%), mucosal sloughing (Figure 3A) in one (10%), and colon mucosal perfusion defect (Figure 2B) was found in seven (70%) patients. Pericolonic stranding (Figure 2C) was identified in eight patients (80%), and pericolonic abscess formation (Figure 3B) was observed in two (20%) patients. Abnormal gas was present in five patients (50%): pneumo-mesocolon in two (40%, Figure 4), and one pa-

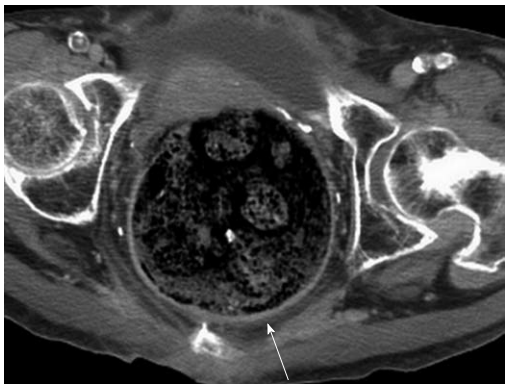

Figure 1 A 70-year-old woman (patient 6) with necrotic stercoral colitis. The computed tomography scan revealed stool impaction and distension of the recto-sigmoid colon with asymmetrical wall thickening at the posterior aspect (arrow).

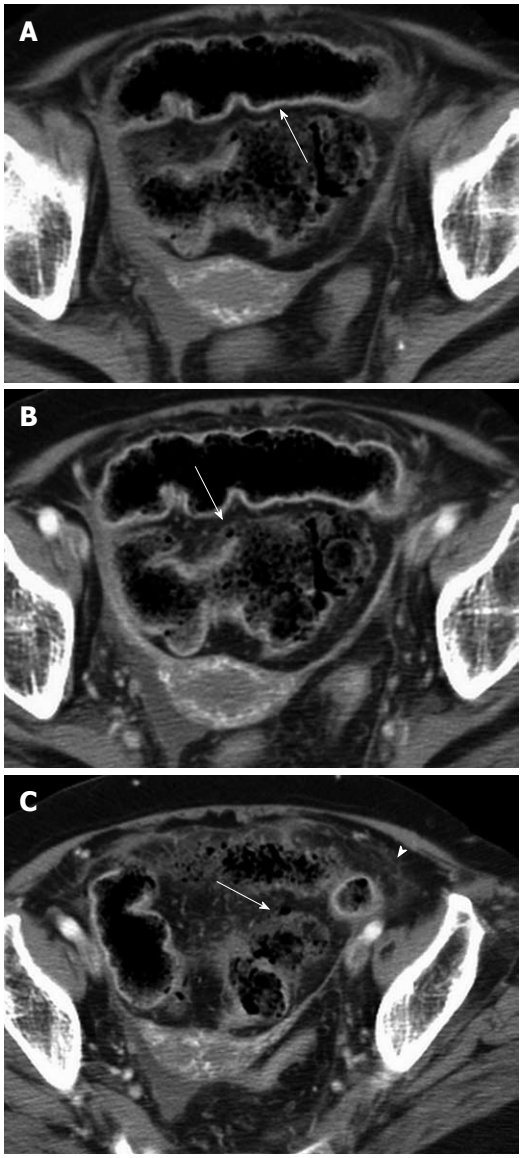

Figure 2 An 80-year-old woman (patient 5) with perforation of the necrotic stercoral colitis at the sigmoid colon. A: An unenhanced computed tomography (CT) scan reveals dense mucosa (arrow) conforming to the colon wall; B: An enhanced abdominal CT scan reveals discontinuation of the colonic mucosa (arrow) suggesting perfusion defect; C: A small air bubble abutting the damaged colon (arrow) and increased pericolonic infiltration (arrowhead) can be seen.

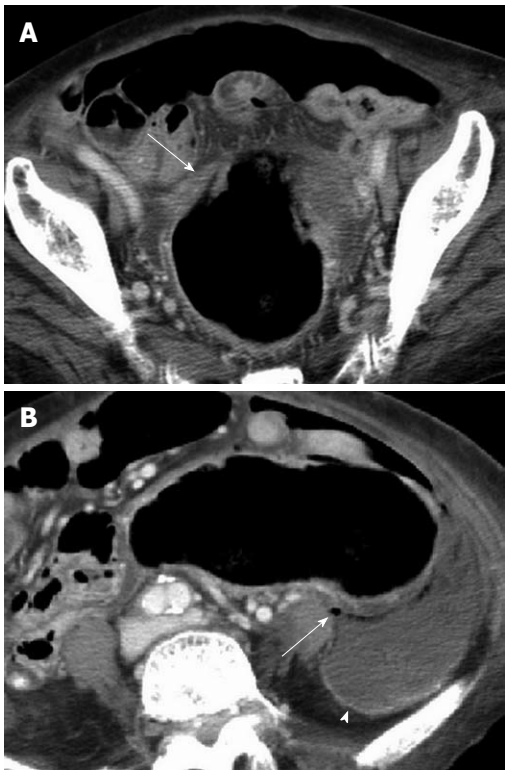

Figure 3 An 88-year-old women (patient 7) with perforation of the necrotic stercoral colitis at the sigmoid colon. A: An enhanced abdominal computed tomography scan reveals mucosal flap (arrow) slough into the lumen of the colon indicating mucosal sloughing; B: Air pockets (arrow) abutting the colonic wall and pericolonic loculated fluid indicative of abscess formation (arrowhead).

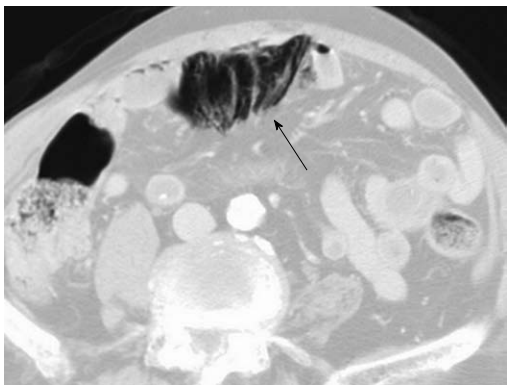

Figure 4 An 87-year-old man (patient 4) with perforated stercoral colitis at the proximal end of co-existing rectosigmoid colon cancer. An enhanced abdominal computed tomography scan at lung-window setting reveals air confined inside the mesocolon indicating pneumo-mesocolon (arrow).

tient (20%) with pneumoperitoneum was identified by radiograph.

Dense mucosa was evaluated with pre-contrast CT scanning in 8 of the patients who had undergone scanning of the lower abdomen. Dense mucosal lining conforming to the colon wall was differentiated from the fecalith which presented as clustered masses in the lumen with a calcified surface and gas in between.

Inter-observer agreement is shown in Table 2. Agreement was good to excellent for all signs except wall thick-

ening and perfusion defect. Disagreement occurred with respect to wall thickening in three patients, perfusion defect in four, dense mucosa in one, mucosal sloughing in one, pericolonic stranding in one, and abnormal gas in one patient. Consensus was generally achieved following an open discussion and the opinion of a third radiologist when necessary.

DISCUSSION

Stercoral ulcer with perforation was first described by Berry in 1894, and to date, fewer than 150 cases have been reported[4]. The incidence of perforated stercoral ulcer at autopsy ranges from 0.04% to 2.3%. Pre-mortem diagnosis is even less frequent, which suggests that the incidence of this condition is often underestimated[3]. One study reported that stercoral perforation of the colon was found in 0.5% of all surgical colorectal procedures, 1.2% of all emergency colorectal procedures, and 3.2% of all colonic perforations[5].

Fecal impaction and perforation occur most often in the sigmoid colon. The sigmoid colon is the narrowest region of the entire colon, and passage of stools with a more solid consistency can be difficult. In such cases, fecaloma exerts localized pressure on the walls of the sigmoid colon, the area with the most precarious vascular supply[6], especially the vascular region known as Sudeck's point. Prolonged localized pressure and ischemia can give rise to pressure ulceration[7,8].

Distention predisposes the colon to insufficient perfusion, leading to slight, moderate, or severe ischemic lesions[9]. Ischemic colitis will occur when intraluminal pressure exceeds 35 cm H_2O for hours[10]. Maurer *et al*[5] have postulated that colonic dilatation and the presence of multiple fecalomas indicate additional stercoral ulceration and carry the risk of secondary perforation. This view was supported by Huang *et al*[11] by visualization of stercoral ulceration during intraoperative colonoscopy.

Chronic constipation (n = 8) and systemic disease (90%, n = 9) were the common clinical problems of the patients in this study, some of them (50%, n = 5) presented with multiple necrotic foci involving long segmental bowel that spanned the territory of the superior and inferior mesenteric arteries. It is probable that long-term systemic disease weakens the colon, while stool impaction causes bowel dilatation and increases wall tension, which worsens perfusion insufficiency and leads eventually to necrosis and potentially to fatal perforation. Unfortunately, the early clinical signs such as fever (20%, n = 2), peritoneal signs (20%, n = 2), and leukocytosis (70%, n = 7) are insufficient to diagnose this severe condition in order to prompt appropriate intervention in these patients.

Obstructive colitis differs from colonic cancer with marginal ulceration at aspects of normal mucosa distal to cancer and, frequently, centimeters immediately proximal to the carcinoma are free of ulceration and inflammation[12]. As an example of this, NSC was diagnosed in our case number 4.

Fecal impaction was present in all our patients and was located mostly at the sigmoid colon (90%, n = 9), which was highly correlated with surgical findings and a result which agrees with other studies. Proximal dilatation was observed in two patients (20%), and was less frequent than we expected. It is possible that the colon could have ruptured prior to the CT scan, thus relieving the luminal pressure. This could also be due to the possible fulminant course which did not allow time for the colon to dilate. None of these two patients with proximal colon dilatation showed abnormal gas that would have indicated whether the colon had ruptured. Probably owing to absence of proximal colon dilatation in NSC, clinicians underestimate the stool impaction.

Colon wall thickening (60%, n = 6) is an indicator of stercoral colitis caused by edema or acute inflammation. Dense mucosa as a result of mucosal hemorrhage has been reported to be a sign of ischemic bowel[13,14]. This was one of the most frequently observed signs of NSC and occurred in 62.5% (5 of 8) of our cases. Mucosal sloughing (10%, n = 1) and perfusion defect (70%, n = 7) indicated status of ischemia progressing to infarct of the colon. The radiologists' disagreement over wall thickening and perfusion defect may have been the result of subtle and localized changes. These findings indicate that the CT signs of NSC are not obvious, and that radiologists must be aware of the signs to make an early diagnosis. Pericolonic fat stranding was the most frequent CT sign of NSC observed in our patients (8 of 10, 80%). Intraoperative findings indicated that pericolonic fat stranding was the result of pericolonic inflammation and edema. The pericolonic reaction was most likely the cause of the intolerable abdominal pain experienced by these patients.

NSC with abnormal gas (50%, n = 5) often appears on CT scans as small gas bubbles in the proximity of the colon wall: pneumo-intestinalis coli or pneumo-mesocolon. This is usually undetected by radiography and differs from gastroduodenal perforation that usually presents massive pneumoperitoneum. Intraoperative findings indicate that the perforation can be temporarily plugged by a fecaloma. In our sample, a standing radiograph was not often obtained, partly because pneumoperitoneum was not suspected clinically, and the elderly patients were usually in a weakened state that impeded their assuming a standing posture. Pneumo-mesocolon was not always evident on the radiograph because it was obscured by the presence of a lot of fecal material in the abdomen. This explains why only one (20%) of five cases with abnormal gas was detected by radiography. Thus, abdominal CT, with meticulous searching for signs of abnormal gas, is required. Pericolonic abscess formation was seen in two (20%) of our patients. When the NSC is perforated, the viscous nature of the fecal material causes it to further impede the peritoneum with soiling.

NSC differs from other colitis by absence of diarrhea clinically. It can be confirmed by intraoperative and histological findings[5]. At surgery, stercoral ulcers and perforations are usually found on the anti-mesenteric side; ulcerations usually have sharp margins and measure 1-10 cm, and are occasionally multiple. Histological findings include

sharp demarcation without undermining at ulcer margins, and transmural necrosis at the perforated site. Treatment is usually resection of the affected bowel, colostomy, and Hartmann's procedure[1,5].

Typically, only the more severe cases in this sample would have been discussed at the conference, and this resulted in a high mortality rate among our patients (70%; 7/10, which is higher than previously reported[1]).

In the elderly and in nursing home patients, ascites associated with liver cirrhosis or malnutrition is often encountered. This could obscure the significance or specificity of pericolonic fluid accumulation for colonic pathology. Thus, we did not investigate this factor for NSC.

This retrospective study consisted of a small population of patients with NSC; thus, the statistical significance and likelihood ratios of each CT sign for NSC could not be determined appropriately. Owing to the nature of the retrospective study, some important clinical data and imaging were unavailable. This study aimed to alert clinicians to the CT findings of NSC, a potentially fatal condition. A further study with a larger number of patients is needed to validate the accuracy of our CT findings.

In summary, elderly patients with a history of chronic constipation and systemic disease, presenting with fecal impaction and acute abdomen with indeterminate leukocytosis, are at risk of NSC. CT is justified to be suggested to investigate the possibility of NSC. Pericolonic stranding, perfusion defect and dense mucosa were the most sensitive CT measures for NSC, detecting about 80%, 70%, and 62.5% of the cases, respectively. Awareness of NSC and familiarity with these CT signs enables us to make a differential diagnosis between this fatal condition and benign stool impaction.

COMMENTS

Background

In clinical practice, fecaloma-related necrotic stercoral colitis (NSC) is an infrequently and easily overlooked disease. It frequently occurs in patients who are elderly or have inactive status. High mortality is encountered when perforation takes place.

Research frontiers

In fact, most fecal impaction is usually relieved by non-invasive management, meaning that surgical and pathologic evidence for NSC has been unobtainable. In this era of liberal computed tomography (CT) use for patients with acute abdomen in emergency departments, few articles about CT of stercoral colitis have been published.

Innovations and breakthroughs

Over the last few years, the authors have collected ten patients with clinical, surgical, pathologic and radiographic evidence of NSC. In practice, clinical clues alone are insufficient to exclude the disease. Characteristic CT presentations are useful to delineate this colon pathology, especially dense mucosa, pericolonic stranding, perfusion defect, and mucosal sloughing of colon.

Peer review

The paper is an interesting one, but it needs major language refinement and some additional information to be provided. I then would suggest acceptation for publication, provided satisfactory revision requirements are met.

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BRIEF ARTICLE

Management of patients with sphincter of Oddi dysfunction based on a new classification

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Abstract

AIM: To propose a new classification system for sphincter of Oddi dysfunction (SOD) based on clinical data of patients.

METHODS: The clinical data of 305 SOD patients documented over the past decade at our center were analyzed retrospectively, and typical cases were reported.

RESULTS: The new classification with two more types (double-duct, biliary-pancreatic reflux) were set up on the basis of the Milwaukee criteria. There were 229 cases of biliary-type SOD, including 192 (83.8%) cases cured endoscopically, and 29 (12.7%) cured by open abdominal surgery, and the remaining 8 (3.5%) cases observed with unstable outcomes. Eight (50%) patients with pancreatic-type SOD were cured by endoscopic treatment, and the remaining 8 patients were cured after open abdominal surgery. There were 19 cases of double-duct-type SOD, which consisted of 7 (36.8%) patients who were cured endoscopically and 12 (63.2%) who were cured surgically. A total of 41 cases were diagnosed as biliary-pancreatic–reflux-type SOD. Twenty (48.8%) of them were treated endoscopically, 16 (39.0%) were treated by open abdominal surgery, and 5 (12.2%) were under observation.

CONCLUSION: The newly proposed SOD classification system introduced in this study better explains the clinical symptoms of SOD from the anatomical perspective and can guide clinical treatment of this disease.

Key words: Sphincter of Oddi dysfunction; Classification; Diagnosis; Treatment

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INTRODUCTION

Sphincter of Oddi dysfunction (SOD) is characterized by a series of clinical pain symptoms caused by abnormalities in sphincter contractility[1]. The sphincter of Oddi (SO), a fibromuscular sheath encircling the distal common bile duct (CBD), pancreatic duct and common channel, controls the flow of bile and pancreatic secretions into the duodenum and prevents reflux of duodenal contents into the pancreaticobiliary system[2]. SO dyskinesia caused by injury or/and inflammation can result in a hypo- or

hypertonic sphincter with altered motility, causing an intermittent functional blockage of the sphincter. As it is often difficult to distinguish SO stenosis from dyskinesia, the term "sphincter of Oddi dysfunction" is used to cover both conditions[3]. For SOD, the Milwaukee classification proposed by Hogan and Geenen has been widely accepted; it classifies SOD into two types: the biliary type and the pancreatic type[4-6]. Biliary and pancreatic SOD are each sub-classified as type Ⅰ, Ⅱ and Ⅲ on the basis of symptoms, laboratory tests and radiological imaging[4,5]. This classification explains the clinical manifestations of SOD from the anatomical perspective and reveals the nature of SOD, thus providing a reliable anatomical basis for appropriate clinical treatment. In our long-term clinical practice, however, we have found this classification system somewhat flawed. For instance, the clinical manifestations in some patients with SOD are difficult to be satisfactorily defined using the Milwaukee criteria. Therefore, the existing system is insufficient in guiding the clinical treatment of all SOD patients. In this paper, we introduced a new classification of SOD based on the Milwaukee classification. In the new classification system, SOD is divided into four types according to anatomy, symptoms, endoscopic tests and radiological imaging. These types include not only those already established by the Milwaukee criteria (biliary and pancreatic types), but also two more new types (the double-duct and biliary-pancreatic reflux types) in an attempt to improve the clinical treatment of SOD.

MATERIALS AND METHODS

Patient data

A total of 1013 patients underwent endoscopic retrograde cholangiopancreatography (ERCP) from January 1999 to January 2009, and 305 (30%) patients who met the diagnostic criteria of SOD were included in this study. In the 305 SOD patients, there were 198 men and 107 women, aged from 36-79 years (mean age, 47.5 years) (Table 1). All the patients had to meet the following diagnostic criteria for SOD: (1) typical clinical symptoms, with or without abnormal liver function and amylase levels[4,5]; and (2) baseline sphincter of Oddi manometry (SOM) pressure > 40 mmHg[7], and/or paradoxical pressure response to cholecystokinin[8], and/or delayed emptying of contrast medium in the common bile duct as indicated by ERCP[9], and/or a diameter of common bile duct ⩾ 10 mm without evidence of stones or tumors[6]. Preliminary classification was performed based on the development of the disease, examinations and treatment, e.g. ERCP, magnetic resonance cholangiopancreatography (MRCP), endoscopic sphincterotomy (EST) and endoscopic pancreatic duct sphincterotomy (EPS). Some patients were ultimately classified according to the findings of surgical exploration. Intraoperative choledochography, choledochoscopy and pancreatoscopy were performed if necessary.

It may still take a long time to establish the diagnosis for double-duct type and biliary-pancreatic reflux type SOD. For SOD of the double-duct type, both the biliary and pancreatic ducts of the sphincter of Oddi are affected. So, the biliary pain and pancreatic pain may occur simultaneously or alternately. For SOD of the biliary-pancreatic reflux type, the patients may have a congenital abnormality in the convergence of the biliary and pancreatic systems. When the ampullary anti-reflux valve is dysfunctional, small amounts of bile may repeatedly reflux into the pancreatic duct, which often induces pancreatitis. That is why sometimes an over long common duct of the biliary and pancreatic duct is found in auxiliary examination and operation.

Table 1 Clinical features of sphincter of Oddi dysfunction patients

Clinical features	*n* (%)
Abdominal pain	
Retrosternal pain	235 (77.05)
Epigastric pain	70 (22.95)
Radiating pain	45 (14.75)
Timing of onset	
After high-fat meals	241 (79.02)
After ordinary meals	21 (6.89)
Uncertain	43 (14.10)
Jaundice	
With	21 (6.89)
Without	284 (93.11)
Liver enzyme levels	
Abnormal	58 (19.02)
Normal	247 (80.98)
Serum amylase	
Abnormal	63 (20.66)
Normal	242 (79.34)
Cholangiectasis	
⩾ 10 mm	289 (94.75)
< 10 mm	16 (5.25)
Pancreatic duct dilation	76 (24.92)

Therapeutic methods

The therapeutic methods and results in patients with different types of SOD are shown in Table 2.

Typical cases

Case 1: A 43-year-old man first suffered from pancreatitis in November 2004. Three months later, his pancreatitis recurred, and B-mode ultrasonography suggested cholecystolithiasis. The patient suffered from recurrent pains in the upper abdomen six months after cholecystectomy performed in March 2005 due to clinically diagnosed biliary pancreatitis. Computed tomography (CT) suggested swelling of the pancreatic head and pancreatic duct distension, accompanied by stones. Moreover, the common bile duct had a diameter of 8 mm. With the duodenum preserved, the patient underwent resection of the swollen head of the pancreas, which was assumed to be compressing the bile duct, resulting in bile duct distension before the surgery. However, three months after surgery, the patient began to experience biliary colic with a frequency of 2-3 times per month. Laboratory tests showed normal blood amylase levels and aggravated liver dysfunction with alkaline phosphatase increasing from 579 to 1858 IU/L and glutamyl transpeptadase from 658 to 2006 IU/L, accompanied by enlargement of the diameter of the common bile duct

Table 2 Detailed classification, treatment and outcomes associated with sphincter of Oddi dysfunction

	n (%)
SOD biliary type	229/305 (75.08)
EST preferred	213/229 (93.01)
Improved	202/213 (94.83)
Good	182/202 (90.10)
Moderate	10/202 (4.95)
Poor	10/202 (4.95)
Failed	11/213 (5.16)
Exploratory laparotomy	29/229 (12.66)
Choledochointestinal anastomosis	21/29 (72.41)
Sphincter of Oddi plasty	8/29 (27.59)
Conservative treatment	8/29 (27.59)
Pancreatic type SOD	16/305 (5.25)
EPS preferred	13/16 (81.25)
Improved	10/13 (76.92)
Good	8/10 (80.00)
Moderate	2/10 (20.00)
Failed	3/13 (23.08)
Pancreatojejunal anastomosis	8/16 (50.00)
Double-duct type SOD	19/305 (6.23)
EST preferred	11/19 (57.89)
Improved	7/11 (63.64)
Failed	4/11 (36.36)
Exploratory laparotomy	12/19 (63.16)
Choledocho-, pancreato-intestinal anastomosis	7/12 (58.33)
Pancreatic head resection, choledocho-intestinal anastomosis	1/12 (8.33)
Duodenopancreatectomy	1/12 (8.33)
Biliary sphincterotomy, pancreato-intestinal anastomosis	3/12 (25.00)
Biliary-pancreatic reflux type SOD	41/305 (13.44)
EST preferred	34/41 (82.93)
Improved	20/34 (58.82)
Failed	14/34 (41.18)
Laparotomic BPD	16/41 (39.02)
Conservative treatment	5/41 (12.16)

SOD: Sphincter of Oddi dysfunction; EST: Endoscopic sphincterotomy; EPS: Endoscopic pancreatic duct sphincterotomy; BPD: Biliopancreatic diversion.

from 8 to 14 mm. Endoscopic bile duct sphincterotomy was performed twice in three months but without satisfactory curative effect. Consequently, an open abdominal surgery was performed at our center for frequent biliary colic. The common bile duct was transected, and then a cholangiojejunostomy was performed. During the two-year follow-up, liver function returned to normal levels and no further abdominal pain was reported by the patient.

Case 2: A 41-year-old man was diagnosed as having acute pancreatitis in June 2001 due to pains in the upper abdomen and an increase in blood amylase levels. The patient was discharged one week later. In October 2001, the patient experienced abdominal pain again and was diagnosed as having pancreatitis from a swollen pancreas head according to CT examination. In addition, B-mode ultrasonography detected small stones in the gallbladder. Laparoscopic cholecystectomy was performed in February 2002. Pancreatitis recurred dozens of times during the period from February 2002 to August 2003. Accordingly, emergent EST was then carried out, with subsequent intraoperative findings including a swollen ampulla of Vater, duodenal hyperemia, and mucosal edema. No bile flow was observed within the ampulla of Vater after a 10-mm incision was cut. This suggested that EST had failed, and ERCP could not be performed. In November 2003, EST was performed again when duodenal hyperemia and edema subsided. ERCP was successful, which indicated a narrow and flexuous common duct of the bile and pancreatic ducts. After a 15-mm incision in the ampulla of Vater was executed, bile flow was observed; however, it was still difficult to insert a tube into the bile duct. Even though bile samples were collected occasionally from the hepatic portal area through a tube for amylase measurement, the amylase level was still as high as 7600 U/L, demonstrating that the previous EST had failed to prevent pancreatitis. During a period of one year after the second EST, pancreatitis recurred eight times in this patient, indicating the failure of the second EST. Biliary-pancreatic shunting was then performed based on evidence of biliary-pancreatic reflux. In January 2005, the patient received transection of the common bile duct and choledochointestinal anastomosis. During the operation, the bile duct pressure was measured to be 12 cmH_2O, and the bile amylase level was 690 U/L. In addition, pancreatoscopy revealed an 11-mm stenotic segment of the common duct after two ESTs, and the common duct was tortuous, which may lead to biliary-pancreatic reflux. During the two-year follow-up, pancreatitis did not recur. Moreover, a CT scan performed one year after the last surgery showed obvious shrinkage of the swollen pancreatic head, indicating an effective relief of the edema.

RESULTS

Based on our clinical observations, we added the double-duct type and the biliary-pancreatic reflux type of SOD to the Milwaukee classification, to achieve greater clarity in the SOD clinical symptoms and to guide the clinical treatment of SOD. The therapeutic methods and effects using this new classification are shown in Table 3.

Biliary type SOD

Among the 305 patients, 229 (75.1%) definitively demonstrated biliary-type SOD, and 6 of them were not eligible for EST due to a diverticulum near the ampulla of Vater; these patients were treated by open abdominal surgery. Of the 213 patients who underwent EST, 202 (94.8%) were successfully treated, but 10 (4.9%) of these patients had unsatisfactory therapeutic effects after EST, with biliary colic and bile duct distension. EST failed in 11 (5.2%) patients. Hence, a total of 21 patients had unsatisfactory therapeutic effects or EST failure. In addition to the 16 patients for whom open abdominal surgery was initially prescribed, only 29 patients actually underwent surgery (choledochointestinal anastomosis in 21 patients and sphincteroplasty in 8 patients) and had good therapeutic effects. The other 8 patients underwent conservative treatment, but showed no stable therapeutic effects.

Table 3 Clinical classification and features of sphincter of Oddi dysfunction

Features	Bile duct type (type Ⅰ)	Pancreatic duct type (type Ⅱ)	Double-duct type (type Ⅲ)	Biliary-pancreatic reflux type (type Ⅳ)
Abdominal pain	Retrosternal pain	Epigastric pain	Ⅰ + Ⅱ	Same as type Ⅱ
Radiating pain	The central point of the back	The left back or indefinite site	Ⅰ + Ⅱ	Same as type Ⅱ
Timing of onset	After high-fat meals	After ordinary meals	Ⅰ + Ⅱ	At night or in the morning
Jaundice	±	-	±	-
Liver enzyme levels	±	-	+	±
Serum amylase levels	-	+	±	+
Bile duct distension	⩾ 10 mm	< 10 mm	⩾ 10 mm	⩾ 10 mm
Pancreatic duct distension	-	++	+	+
Anterograde cholangiography Voiding time	> 10 min	Normal	> 10 min	Unexpected pancreatic duct visualization

Pancreatic type SOD

There were 16 (5.3%) cases of the pancreatic type of SOD, and 3 of them had multiple stones that were difficult to remove thoroughly because they were located deep in the pancreatic duct. Therefore, EPS was not indicated for these 3 patients. The other 13 cases were first treated by EPS, but the treatment failed in 3 cases (a success rate of 76.9%). Hence, a total of 6 cases were managed with pancreatico-jejunostomy subsequently. The therapeutic effect was satisfactory in these cases. The other 10 patients treated by EPS (5 patients with pancreatic duct stent placement) were followed up for more than two years. Satisfactory therapeutic effects were exhibited in 8 (73%) patients, and the other 2 patients were then treated by subsequent open abdominal surgery. These cases suggest that open abdominal surgery may be helpful for treating SOD regardless of whether pancreatic duct stones are involved.

Double-duct type SOD

There were 19 (6.2%) cases of double-duct type SOD, in which both the bile duct and the pancreatic duct of the sphincter of Oddi were affected. Among them, 11 patients were treated by EST, and 7 (63.6%) were cured. The remaining 8 cases were treated surgically. A total of 12 cases were treated by open abdominal surgery. Seven patients underwent choledocho-intestinal anastomosis plus pancreato-intestinal anastomosis, and one was treated with a resection of the pancreatic head with the duodenum preserved, followed by choledocho-intestinal anastomosis. Duodenopancreatectomy was performed on one patient. The other three received biliary sphincterotomies by EST and pancreato-intestinal anastomosis to treat distension of the pancreatic duct. Follow-up results revealed good curative effects in these cases.

Biliary-pancreatic reflux type SOD

Forty-one cases (13.4%) of SOD met the profile of the biliary-pancreatic reflux type, and EST was performed in 34, with a success rate of 58.8% (20/34). Surgical treatment was preferentially carried out for 2 cases, and then, a total 16 cases underwent laparotomic biliopancreatic diversion (BPD). During a follow-up period of 0.5-7 years, 14 patients showed satisfactory therapeutic effects, and in the other 2 cases, sclerotic changes were observed throughout the pancreas. After surgery, abdominal pain occurred occasionally and was significantly mitigated in these 2 cases. Five cases without receiving any treatment are under observation, and their symptoms still often occurred.

DISCUSSION

Although the Milwaukee classification system has been widely accepted for the classification of patients with suspected SOD, it has some potential problems. For example, the description of typical biliary or pancreatic pain may be interpreted differently by different doctors, which may lead to inappropriate referrals for SOM. In addition, according to the Milwaukee criteria, a CBD diameter of at least 12 mm is required for the diagnosis of SOD. Most patients being investigated for SOD have had their gallbladder removed, and in the past, it was believed to be normal for a post-cholecystectomy CBD to be dilated by 2-3 mm[10]. Moreover, there is a question of whether patients with both biliary and pancreatic pain should be classified into the biliary type or the pancreatic type. Freeman *et al*[11] stated that for this group, all patients should undergo biliary sphincterotomy, and 40% should have pancreatic sphincterotomies. How to interpret these clinical data? The Milwaukee classification has some limitations. In our study, there were 60 cases that could not be accurately interpreted by the Milwaukee classification criteria. Our long-term observation of the clinical cases suggests that our new classification based on anatomy, symptoms, endoscopic tests and radiological imaging is superior to the Milwaukee criteria in guiding the treatment of SOD. According to the Milwaukee criteria, the two types of SOD (biliary and pancreatic) can be further classified into three subtypes each, making classification complex. The newly proposed classification of SOD and the clinical characteristics associated with each type are listed in Table 3.

The new classification system presented in this paper is simpler than the initial one, but continues to closely follow the Milwaukee classification criteria. For example, biliary-type SOD patients only have biliary pain, and pancreatic-type SOD patients only have pancreatic pain. The two types are no longer divided into subtypes. MRCP usually shows the distension of bile ducts for biliary SOD and dilation solely of the pancreatic duct for pancreatic-

type SOD. With respect to treatment, EST can often yield better results for patients suffering from biliary SOD, and EPS should be a good choice of treatment for patients with pancreatic SOD.

We have paid more attention to the clinical characteristics and significance of the other two types of SOD, i.e. the double-duct and biliary-pancreatic reflux types.

In double duct type of SOD, both the biliary and pancreatic ducts of the sphincter of Oddi are affected. Clinical cases of SOD meeting these criteria have previously been reported but have not been definitively classified[12]. The characteristics of this SOD type include symptoms typical of both biliary- and pancreatic-type SOD that appear simultaneously or alternately, with mobile positions of abdominal pain and radiating pain. Meanwhile, laboratory tests indicate elevated levels of liver-related enzymes and amylase in the blood. These findings usually result in the diagnosis of chronic biliary pancreatitis, manifested as mild abnormal liver function due to edema of the pancreatic head. Imaging exams usually show distension of both the bile and pancreatic ducts and stones in the pancreatic duct. Notably, frequently recurrent pancreatitis and evident distension of the pancreatic duct could mislead surgeons to focus on pancreatitis, thus underestimating the severity of mild bile duct distension, causing the neglect of possible SOD diagnoses, and resulting in unsuccessful treatment. Therefore, it is necessary to outline the double-duct type of SOD so that patients with these symptoms can be effectively treated. Case 1 is a typical SOD of the double-duct type, with mild bile duct distension due to sphincter of Oddi stenosis, and not due to compression by the head of the pancreas. This was proved by the finding that the obstruction in the extremity of the bile duct was not relieved even when the head of the pancreas was resected in the first operation. In patients with the double-duct type SOD, the sphincter of common duct is short, but the inferior stenotic segment of the bile duct is relatively long. Hence, patients with this form of SOD can be treated by surgery rather than EPS.

The anatomical basis for biliary-pancreatic reflux type of SOD is probably as follows: Patients may have a congenital abnormality in the convergence of the biliary and pancreatic systems, i.e. an overlong duct (> 11 mm). Inflammation and stenosis mainly occur in the sphincter of the common duct, whereas the sphincters of the superior bile duct and the pancreatic duct remain relatively normal or only mildly affected. If fibrosis of the ampullary septum causes dysfunction in the anti-reflux valve, reflux between the bile and pancreatic ducts is likely to occur. Repeated reflux of small amounts of bile into the pancreatic duct will usually induce pancreatitis, including recurrent chronic biliary pancreatitis and even severe acute pancreatitis. Although the clinical manifestations of this SOD are quite similar to those of the pancreatic duct type, there are a few differences: (1) As the reciprocal reflux between the fluids in the bile and pancreatic ducts is structurally barrier-free, the pancreatic duct orifice has no evident stenosis or obstruction, and so distension of the pancreatic duct does not occur; (2) Because there was only mild biliary-pancreatic reflux in most cases, the pancreatic duct and gland alveoli in the head of the pancreas near the biliary-pancreatic convergence were usually affected, inducing swelling of the pancreatic head, as indicated by imaging examination. Therefore, these cases are sometimes diagnosed as pancreatitis with a pancreatic head mass. Some authors have reported tumor-like features of chronic pancreatitis, and some of these cases may suffer from biliary-pancreatic reflux[13,14]; (3) In this type of SOD, EST failed to incise the stenotic segment of the sphincter of Oddi due to the slender and tortuous common duct. EST was successful in only 56% of cases with this type of SOD at our center. However, the achievement ratio of incision on the normal ampulla of Vater was nearly 100%[15,16]. Moreover, some patients can only be treated by open abdominal surgery due to failure to incise the stenotic common duct after several attempts by EST; and (4) The amylase levels in bile sampled from the biliary tract during ERCP in biliary-pancreatic-reflux-type SOD patients were about 2-10 times higher than normal.

Although distension does not always occur in the pancreatic duct and is usually slight in the biliary duct in the early stages of biliary-pancreatic reflux SOD, the symptoms of pancreatitis may be more serious than those in the simple pancreatic-type SOD. Because the common duct of the bile and pancreatic ducts is simply a potential duct due to the tension of the sphincter of Oddi, an image of an over long common duct was obtained from ERCP or MRCP in only a few cases. Case 2 mentioned above illustrates that the therapeutic effects of BPD were satisfactory for the biliary-pancreatic-reflux-type SOD patients who failed in EST. This demonstrates the significance of using the biliary-pancreatic reflux type classification for SOD.

In summary, the new classification of SOD proposed in this study demonstrates significant advantages for guiding the diagnosis and treatment of SOD patients in China, as compared with the conventional Milwaukee criteria. Nonetheless, further investigations on the applicability of this quaternary classification system to patients in other regions are needed.

COMMENTS

Background

Sphincter of Oddi dysfunction (SOD) is a pathological syndrome that is usually classified into the biliary type or the pancreatic type according to the Milwaukee criteria. However, this classification has some drawbacks in clinical practice, some of which result in flawed classification and failure to properly guide diagnosis and treatment.

Research frontiers

The conventional SOD classification system is insufficient in guiding the clinical treatment of all SOD patients. The authors conducted a retrospective analysis of 305 patients with SOD according to the clinical records in the past 10 years, and proposed a modified classification system based on the Milwaukee classification, which includes all SOD symptoms.

Innovations and breakthroughs

The authors proposed a new classification system for SOD according to anatomy, symptoms, endoscope tests and radiological imaging, i.e. SOD is divided into four types instead of two types: the biliary-type, the pancreatic-type, the double-duct type and the biliary-pancreatic reflux type. The new classification demonstrates significant advantages for guiding the diagnosis and treatment of SOD patients in China, as compared with the conventional Milwaukee criteria.

Applications

The new classification system has significant advantages for guiding the diagnosis and treatment of SOD patients, thus improving the clinical treatment of SOD.

Peer review

In order to solve some problems of the Milwaukee classification of SOD, the authors have proposed a new interesting classification. Compared with the Milwaukee classification, the new classification system presented in this paper is simpler than the Milwaukee classification, better explains clinical symptoms of the disease from the anatomical perspective, and should have some application values in guiding the diagnosis and treatment of SOD.

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BRIEF ARTICLE

Effect of preoperative biliary drainage on malignant obstructive jaundice: A meta-analysis

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Abstract

AIM: To evaluate the effect of preoperative biliary drainage (PBD) on obstructive jaundice resulting from malignant tumors.

METHODS: According to the requirements of Cochrane systematic review, studies in the English language were retrieved from MEDLINE and Embase databases from 1995 to 2009 with the key word "preoperative biliary drainage". Two reviewers independently screened the eligible studies, evaluated their academic level and extracted the data from the eligible studies confirmed by cross-checking. Data about patients with and without PBD after resection of malignant tumors were processed for meta-analysis using the Stata 9.2 software, including postoperative mortality, incidence of postoperative pancreatic and bile leakage, abdominal abscess, delayed gastric emptying and incision infection.

RESULTS: Fourteen retrospective cohort studies involving 1826 patients with malignant obstructive jaundice accorded with our inclusion criteria, and were included in meta-analysis. Their baseline characteristics were comparable in all the studies. No significant difference was found in combined risk ratio (RR) of postoperative mortality and incidence of pancreatic and bile leakage, abdominal abscess, delayed gastric emptying between patients with and without PBD. However, the combined RR for the incidence of postoperative incision infection was improved better in patients with PBD than in those without PBD ($P < 0.05$).

CONCLUSION: PBD cannot significantly reduce the postoperative mortality and complications of malignant obstructive jaundice, and therefore should not be used as a preoperative routine procedure for malignant obstructive jaundice.

Key words: Malignant obstructive jaundice; Preoperative biliary drainage; Meta-analysis; Mortality; Incidence of complications

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

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INTRODUCTION

Surgery for patients with malignant obstructive jaundice carries an increased risk of postoperative complications

and a high mortality rate[1,2] and preoperative hyperbilirubinemia is considered an important risk factor for postoperative complications and death. Hyperbilirubinemia due to obstructive jaundice damages hepatic function, clearance of circulating endotoxins, coagulation system, immune function, and gastrointestinal barrier[3-6]. To avoid the poor outcome, preoperative biliary drainage (PBD) has been used to reduce the postoperative morbidity and mortality of these patients. However, PBD has also many drawbacks, such as biliary stent-induced bacterial contamination and risk of cholangitis due to clogging. In addition, biliary stenting generates a severe inflammatory response in the bile duct which may increase the risk of bile leakage at the biliodigestive anastomosis. Since 1970s, several randomized and retrospective studies have compared the effect of PBD and surgery without PBD on malignant obstructive jaundice[7,8]. However, it is difficult to find evidence that routine PBD improves the outcome of patients with malignant obstructive jaundice in clinical practice.

Despite the scarcity of clinical evidence, most patients with malignant obstructive jaundice undergo either percutaneous transhepatic or internal PBD in many centers. This meta-analysis was to evaluate the effect of PBD on malignant obstructive jaundice.

MATERIALS AND METHODS

Search strategy and selection criteria

Studies on obstructive jaundice in the English language were retrieved from MEDLINE and Embase databases from 1995 to 2009 with the key word "preoperative biliary drainage". The primary selection criteria for meta-analysis included patients with malignant obstructive jaundice, those with or without PBD, and those with their postoperative mortality and incidence of complications assessed. The exclusion criteria were patients who underwent different surgical procedures, and those with other severe diseases unrelated to obstructive jaundice. The included studies were reviewed by two independent reviewers, with disagreements settled by group discussion.

Data extraction

Data were independently extracted by two investigators in a standard form. The concordance rate between the two investigators was 100%. Following information was extracted from all included publications including study group, year, number of included patients, type of drainage, postoperative mortality, incidence of postoperative pancreatic and bile leakage, abdominal abscess, delayed gastric emptying and incision infection

Analysis of methodological quality

Methodological quality was analyzed as previously described[8].

Statistical methods

Stata 9.2 software was used in meta-analysis of the data. Effect measures of interest were relative risks for cohort studies and corresponding 95% CI. Estimates of intervention effect on malignant obstructive jaundice were expressed as relative risks using a fixed effect model. χ^2 test or Fisher's extract test was used to calculate the probability values when appropriate. Pooled effect was estimated using a random-effect model. Publication bias was evaluated by funnel plots and Egger test.

Table 1 Characteristics of 14 studies included in this study

Study	Yr	Type of drainage	Patients (*n*)
Hochwald *et al*[9]	1999	Internal and external	71
Martignoni *et al*[10]	2001	Internal and external	30
Pisters *et al*[11]	2001	Internal and external	255
Srivastava *et al*[12]	2001	Internal and external	95
Hodul *et al*[13]	2003	Internal	212
Pešková *et al*[14]	2005	Internal	304
dos Santos *et al*[15]	2005	Internal	53
Tsai *et al*[16]	2006	Internal and external	303
Barnett *et al*[17]	2006	Internal	104
Bhati *et al*[18]	2007	Internal	50
Choi *et al*[19]	2008	Internal and external	49
Ferrero *et al*[20]	2009	Internal and external	60
Abdullah *et al*[21]	2009	Internal and external	82
Li *et al*[22]	2009	Internal and external	140
Total			1826

RESULTS

Fourteen retrospective cohort studies that were relevant and eligible were retrieved according to the selection and exclusion criteria (Table 1). Of the 1826 patients with malignant obstructive jaundice included in the studies, 1028 were subjected to PBD and 798 were subjected to surgery but not to PBD. Furthermore, only internal PBD was used in 5 out of the 14 studies, and both internal and external PBD were described in the other 9 studies.

Overall mortality

No significant difference was observed in postoperative death rate reported in 9 studies between patients with or without PBD [risk ratio (RR) = 0.996, 95% CI: 0.669-1.484, Figure 1].

Incidence of postoperative pancreatic leakage

The incidence of postoperative pancreatic leakage was reported in 10 studies (Figure 2A). In our study, PBD intervention did not reduce the incidence of pancreatic leakage in patients without PBD (RR = 0.792, 95% CI: 0.478-1.311).

Incidence of postoperative bile leakage

The incidence of postoperative bile leakage was reported in 10 studies with no significant difference observed between experimental and control groups (RR = 0.935, 95% CI: 0.576-1.518, Figure 2B).

Incidence of postoperative incision infection

The incidence of postoperative incision infection was reported in 9 studies. A significant difference was observed

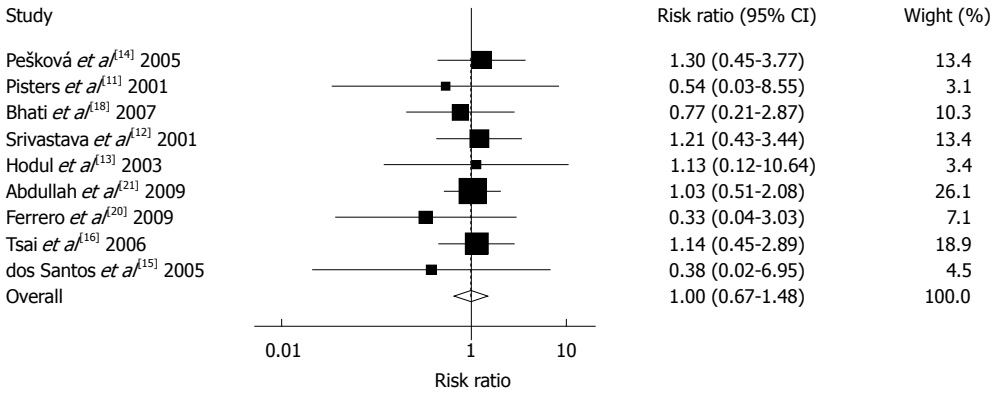
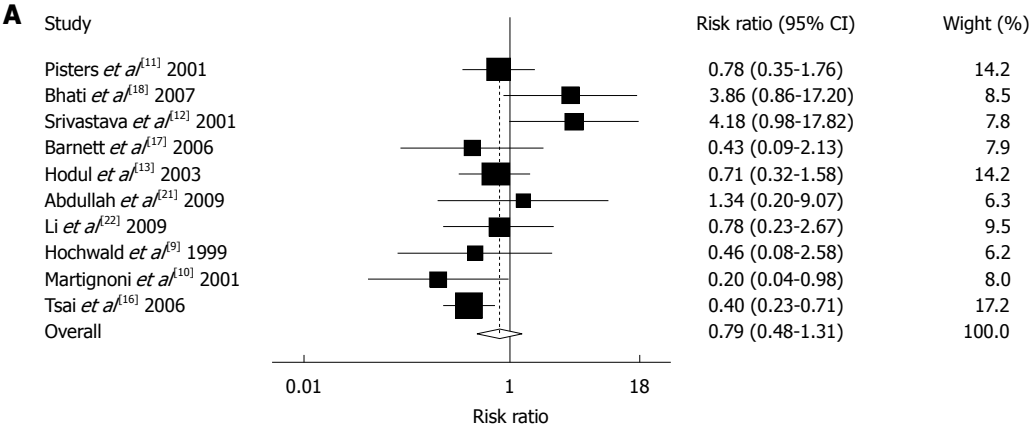


Figure 1 Overall mortality of patients with or without preoperative biliary drainage. Estimates of preoperative biliary drainage (PBD) effects of each study are presented on a log scale along with the 95% CI. The weight of each study is reflected by the size of square. The open diamond represents the global estimate of the PBD effect along with the 95% CI (random-effects model).

A



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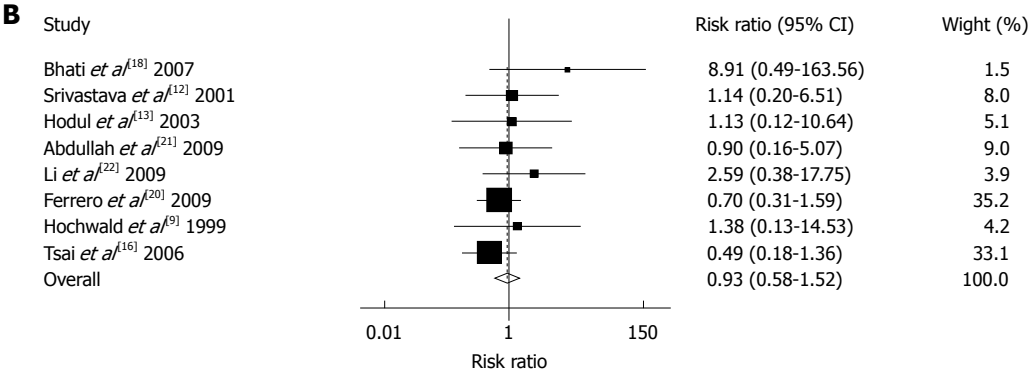

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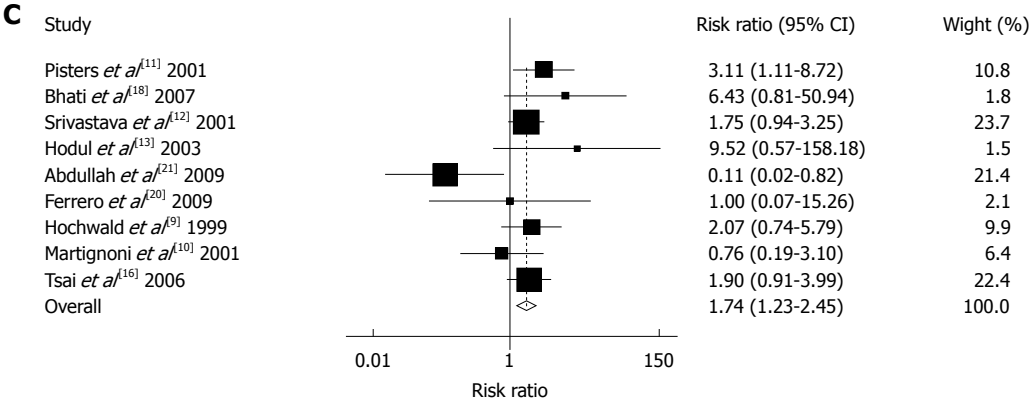

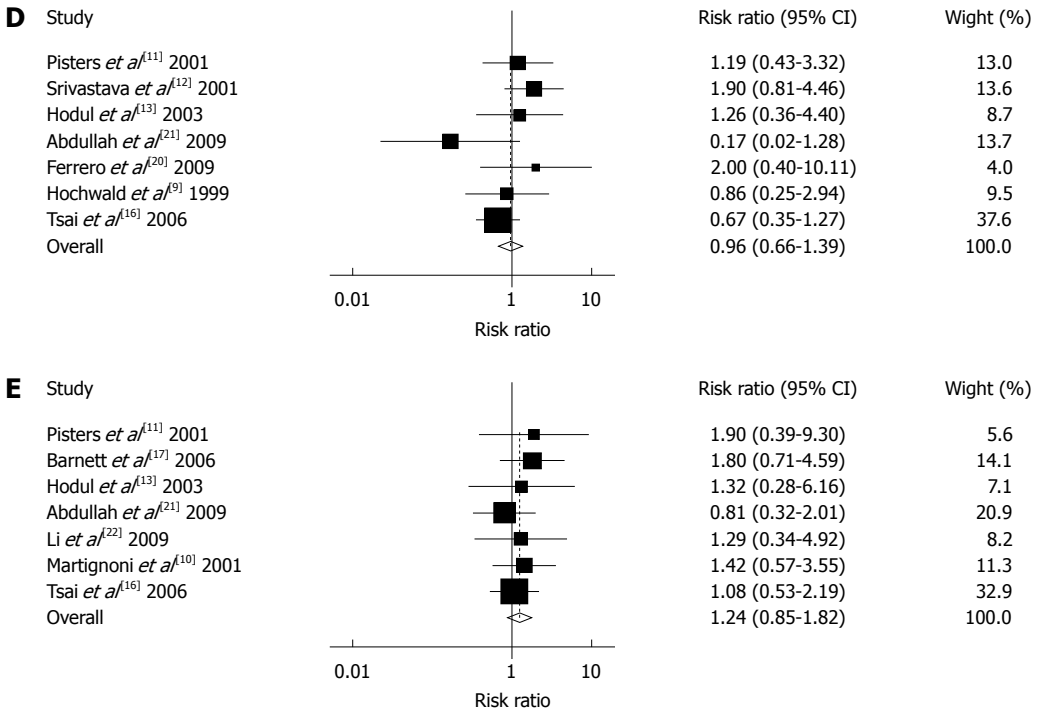

Figure 2 Incidence of postoperative pancreatic leakage (A), postoperative bile leakage (B), postoperative incision infection (C), postoperative abdominal abscess (D), and postoperative delayed gastric emptying (E). Estimates of preoperative biliary drainage (PBD) effect in each study are presented on a log scale along with the 95% CI. The weight of each study is reflected by the size of square. The open diamond represents the global estimate of the PBD effect along with the 95% CI (random-effects model).

between surgical patients with or without PBD (RR = 1.736, 95% CI: 1.229-2.450, $P < 0.05$, Figure 2C).

Incidence of postoperative abdominal abscess

The incidence of postoperative abdominal abscess in patients with or without PBD was reported in 9 studies. PBD could not decrease the postoperative abdominal abscess compared with surgery (RR = 0.957, 95% CI: 0.658-1.392, Figure 2D).

Incidence of postoperative delayed gastric emptying

The incidence of delayed postoperative gastric emptying was reported in 7 studies with no significant difference observed between patients with or without PBD (RR = 1.242, 95% CI: 0.849-1.819, Figure 2E).

DISCUSSION

Biliary obstruction has been identified to be an important risk factor for tumor which may result in alterations of glycogen metabolism, impaired hepatic and renal functions, decreased cell-mediated immunity, increased circulating endotoxins, and depressed synthesis of homeostasis factors[23,24]. These factors can decrease the tolerance of patients to anesthesia and surgery, leading to increasing operative risks. For these reasons, in 1935, Whipple *et al*[25] performed a staged surgery with a preliminary bypass to reduce jaundice and improve hepatic functions. In 1978, Nakayama *et al*[26] found that the operative mortality is significantly reduced after PBD. Since then, more and more investigators have accepted the concept that PBD can improve the hepatic functions of patients with malignant obstructive jaundice[27-31].

With the great advances in surgical techniques and perioperative management, the postoperative complication rate has been dramatically declined in recent years. Whether PBD is still valuable in surgery for malignant obstructive jaundice is questioned by many experts. Several prospective randomized and retrospective studies compared the effect of PBD with surgery without PBD on malignant obstructive jaundice and showed that PBD cannot improve the postoperative outcome but can increase the overall complication rate[32-35]. Although the controversy involves the indication of PBD for malignant obstructive jaundice, some centers still believe that PBD can improve the outcome for some time. To date, whether PBD should routinely be performed for malignant obstructive jaundice is still in debate. One of the reasons why the reported results are distinct is that the overwhelming majority of clinical trials were retrospective and some included heterogeneous groups of patients as well as a variety of different surgical procedures. Thus, unrecognized bias and differences in selection of patients may have affected the results. Another reason is that PBD failing to benefit patients with malignant obstructive jaundice may have a relatively short length of drainage, usually 2-3 wk. In fact, proliferation and fibrosis of bile duct epithelium may take 4-6 wk to recover, and avoid postoperative complications and impaired liver metabolism.

In the present study, the postoperative mortality, the

incidence of postoperative pancreatic and bile leakage, abdominal abscess, and delayed gastric emptying were not significantly different in patients with or without PBD, whereas the incidence of postoperative incision infection was significantly different in patients with or without PBD, which is consistent with other reports[33,36,37]. Povoski *et al*[33] reviewed the effect of PBD and found that PBD has no beneficial effect on the postoperative outcome. In contrast, Trede *et al*[36] showed that the postoperative morbidity is significantly reduced in patients after internal PBD following pancreaticoduodenectom. Lygidakis *et al*[37] also reported that the postoperative morbidity of obstructive jaundice is significantly decreased after internal PBD following pancreaticoduodenectomy. Due to the selective bias in choice of PBD, a well-selected subgroup of patients may benefit from PBD. Moreover, most patients with PBD, experiencing other serious diseases secondary to biliary obstruction, are in a relatively poorer condition than those undergoing surgery. Experimental and clinical evidence has shown that external PBD cannot improve the outcome of surgery, while internal PBD may have a beneficial effect because it can restore the nutritional and immune function[7]. Since internal PBD can significantly reduce the number of postoperative laparotomies for bleeding, anastomotic leakage and abscess, many centers support the view that internal PBD reduces the morbidity rate of obstructive jaundice in patients undergoing surgery. However, Lai *et al*[38] did not support the routine use of internal PBD because of procedure-related complications, mainly cholangitis. In addition, biliary drainage for a proximal tumor with intrahepatic stenosis of the bile duct is also different from that for a distal obstruction. Therefore, various confounding factors affecting the prognosis should be taken into consideration in future clinical investigations.

In conclusion, there is no convincing evidence that supports the view that routine PBD improves postoperative outcome in patients with malignant obstructive jaundice. PBD has its own complications that partially cancel out its benefits. More randomized controlled trials are needed to identify patients who may benefit from PBD.

COMMENTS

Background

Preoperative hyperbilirubinemia in patients with malignant obstructive jaundice is considered an important risk factor for postoperative complications and death. Therefore, preoperative biliary drainage (PBD) has been used to reduce postoperative morbidity and mortality of such patients.

Research frontiers

Since 1970s, several randomized and retrospective studies have compared the effect of PBD with non-PBD on malignant obstructive jaundice. However, it is difficult to find convincing evidence that routine PBD can improve the outcome of patients with malignant obstructive jaundice in clinical practice.

Innovations and breakthroughs

To date, there is no convincing evidence that supports the view that routine PBD improves postoperative outcomes of patients with malignant obstructive jaundice. This is the first meta-analysis of the recent studies concerning the effect of PBD on malignant obstructive jaundice.

Applications

The present meta-analysis indicated that PBD could not significantly reduce the postoperative mortality and the complications of malignant obstructive jaundice. Therefore, PBD may not be regarded as a preoperative routine measure for malignant obstructive jaundice.

Peer review

A meta-analysis of the effect of PBD on malignant obstructive jaundice was performed by reviewing the publications between 1995 and 2009. The authors drew a conclusion that PBD may not be regarded as a preoperative routine measure for malignant obstructive jaundice. This is a very interesting topic for hepatologists and other digestive experts.

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BRIEF ARTICLE

Adjuvant radiotherapy for gallbladder cancer: A dosimetric comparison of conformal radiotherapy and intensity-modulated radiotherapy

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Abstract

AIM: To assess the efficacy and toxicity of conformal radiotherapy (CRT) and compare with intensity-modulated radiotherapy (IMRT) in the treatment of gallbladder cancer.

METHODS: Between November 2003 and January 2010, 20 patients with gallbladder cancer were treated with CRT with or without chemotherapy after surgical resection. Preliminary survival data were collected and examined using both Kaplan-Meier and actuarial analysis. Demographic and treatment parameters were collected. All patients were planned to receive 46-56 Gy in 1.8 or 2.0 Gy per fraction. CRT planning was compared with IMRT.

RESULTS: The most common reported acute toxicities requiring medication (Radiation Therapy Oncology Group, Radiation Therapy Oncology Group Grade 2) were nausea (10/20 patients) and diarrhea (3/20). There were no treatment-related deaths. Compared with CRT planning, IMRT significantly reduced the volume of right kidney receiving > 20 Gy and the volume of liver receiving > 30 Gy. IMRT has a negligible impact on the volume of left kidney receiving > 20 Gy. The 95% of prescribed dose for a planning tumor volume using either 3D CRT or IMRT planning were 84.0% ± 6.7%, 82.9% ± 6.1%, respectively ($P > 0.05$).

CONCLUSION: IMRT achieves similar excellent target coverage as compared with CRT planning, while reducing the mean liver dose and volume above threshold dose. IMRT offers better sparing of the right kidney compared with CRT planning, with a significantly lower mean dose and volume above threshold dose.

Key words: Gallbladder cancers; Adjuvant treatment; Surgery; Radiation therapy

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INTRODUCTION

Gallbladder cancer is the fifth most common malignancy of the gastrointestinal tract[1]. In general, the prognosis of patients with gallbladder cancers is poor, with an overall 5-year survival rate of less than 10%[2]

Gallbladder cancer was associated with a uniformly poor prognosis due to its highly aggressive behavior, only 10%-30% of the patients had resectable tumors at presentation[3]. Better understanding of the biological behavior of the disease, its pattern of dissemination, better diagnostic tools, and more aggressive therapy has resulted in some improvement in survival in the last decade[4]. However, most long-term survivors are patients with incidentally diagnosed carcinomas confined to the mucosa of the gallbladder.

Surgical therapy is the standard treatment for patients presenting with resectable disease. Unfortunately, a large number of patients develop recurrent diseases after undergoing curative resection. The long-term outcome of the patients with recurrent gallbladder cancer is very poor. And local-regional failure is common and is a major cause of mortality[5]. Because of this, adjuvant therapy has been used to improve loco-regional control and survival rate. Several studies have reported improvement in survival for patients treated with adjuvant chemoradiation[6,7].

Actually, the definite role of adjuvant therapy after curative resection is still uncertain. Studies to improve the loco-regional control rates with adjuvant radiation with/without chemotherapy or chemotherapy alone, have been conducted. Although gallbladder cancer is considered to be radiation resistant, radiation has been administered in the form of external beam radiotherapy, intra-operative radiation therapy and brachytherapy[8-11]. However, the relative rarity of this malignant disease made it difficult to conduct large phase Ⅲ studies to guide management in both the adjuvant and comprehensive treatment. This study reports a single-institution series that used adjuvant radiation therapy with or without chemotherapy after resection in patients with locally advanced gallbladder carcinoma. The aim of the study was to evaluate conformal radiotherapy (CRT) and intensity-modulated radiotherapy (IMRT) planning parameters in the treatment of this malignancy.

MATERIALS AND METHODS

Between November 2003 and January 2010, a total of 20 patients with pathologically diagnosed primary adenocarcinoma of gallbladder were treated in the Department of Radiation Oncology in our hospital. All patients were diagnosed with adenocarcinoma of gallbladder. All patients had complete resection, with negative microscopic margins. Demographic data were collected regarding patient age, gender, histological classification, tumor staging (Table 1). In addition to radiotherapy, the majority of patients received concurrent fluoropyrimidine-based and oxaliplatin-based chemotherapy. Concurrent and adjuvant chemotherapy regimens are shown in Table 2. Six patients received postoperative radiotherapy alone. No clear pattern of chemotherapy or standardized dosing regimen was evidenced from chart data available for review. All patients were simulated on a computed tomography-scanner (Siemens Definition AS 40) and were imaged using a slice of thickness 3.0 mm. All simulations were performed using a timed bolus of non-ionic intravenous contrast media to acquire images of early arterial/portal venous contrast phase, and a secondary venous contrast phase. Digital imaging and communications in medicine data were transferred to an inverse IMRT treatment planning station (Philips Pinnacle^3 7.6C). Gross target volumes and clinical target volumes (CTVs) according to ICRU 62 definitions[12] were delineated on a slice-by-slice basis. External beam radiation therapy fields generally encompassed the tumor bed and regional lymph nodes (porta hepatis, celiac, pancreaticoduodenal) to a dose of 45 Gy in 1.8-2.0 Gy daily fractions. Reduced fields to tumor bed plus a 2-2.5 cm margin received an additional 5.0-10.0 Gy. A variety of multi-beam

Table 1 Patient and tumor characteristics (n = 20)

Characteristics	n (%)
Median age (range, yr)	56 (33-73)
Gender	
Male	8 (40)
Female	12 (60)
ECOG performance status	
0	2 (10)
1	16 (80)
2	2 (10)
Tumor and node status	
PT1	0 (0)
PT2	13 (65)
PT3	6 (30)
PT4	1 (5)
NX	
PN0	13 (65)
PN	7 (35)
Tumor grade	
Well	8 (40)
Moderate	9 (45)
Poor	3 (15)

ECOG: Eastern Cooperative Oncology Group; NX: Node staging.

Table 2 Treatment details for the patients

Treatment	No. of patients
CT concurrent RT	9
5-fluorouracil	4
Oxaliplatin	5
Concurrent RT followed by CT	7
5-fluorouracil + oxaliplatin	4
Gemcitabine + oxaliplatin	3
RT followed by CT	3
5-fluorouracil + oxaliplatin	1
Gemcitabine + oxaliplatin	2
Postoperative CT before RT	2
5-fluorouracil bolus	
RT alone	6

Nine patients received concurrent radiochemotherapy, 7 of them received adjuvant chemotherapy. CT: Chemotherapy; RT: Radiotherapy.

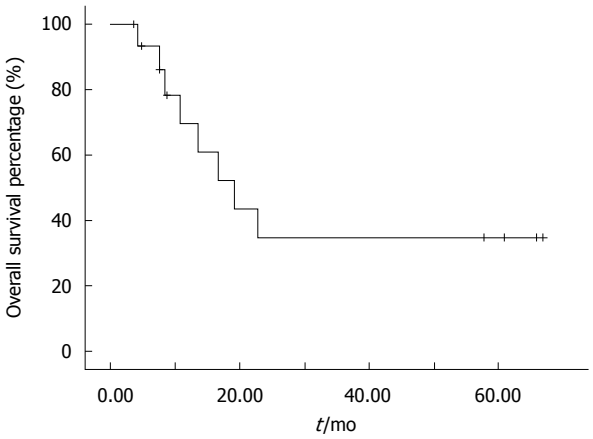

Figure 1 Overall survival for all the patients.

techniques were used to treat the tumor bed to a median total prescription dose of 52 Gy (range, 46-56 Gy). Organs-at-risk (OAR) delineated included the spinal cord, kidneys, and healthy liver. A planning tumor volume (PTV) was created by volumetric expansion of the CTV by 10-15 mm. Dose volume histograms (DVHs) were utilized to evaluate the plans. Prescribed doses to the initial PTV ranged from 46 to 56 Gy in daily doses of 1.8-2.0 Gy. Relative constraints included left kidneys constrained to a D100 of < 20 Gy and D66 < 18 Gy; right kidneys were specified to achieve D100 < 30 Gy, with D66 < 20 Gy. The total mean liver dose was specified to < 22 Gy, and liver V20 kept under 33%. Treatment was delivered using a 10 MV linear accelerator (Siemens Primus M) with 200 MU/min delivery capability using a multi-leaf collimator. Survival data were collected and examined using the Kaplan-Meier method.

By a comparison of CRT and IMRT, all fields of the patients were coplanar. DVHs were obtained for the PTV, kidneys, liver and spinal cord. Acute toxicity was scored using the Radiation Therapy Oncology Group (RTOG) morbidity scoring criteria[13]. Dosimetric endpoints for the target and critical structures were compared using the two tailed paired *t* test.

RESULTS

Survival analysis

The median follow-up for patients alive at analysis was 14.0 mo (range, 3.0-66.9 mo). Nine patients were alive. The median preliminary survival from diagnosis in the 20 patients was 19.2 mo (range, 4.2-66.9 mo, 95% confidence interval: 10.1-28.3). Kaplan-Meier analysis revealed an estimated one-year survival rate of 40.48% (Figure 1).

Toxicity analysis

Twenty patients completed their planned course of treatment without breaks and no reduction of planned chemotherapy. For gastrointestinal (GI) toxicity analysis, radiotherapy or chemotherapy with CRT was well tolerated without > grade 3 acute GI toxicity occurring during radiotherapy, and 11/20 and 4/20 patients reported grade 1 upper and lower acute RTOG GI toxicity scores. The most common reported acute toxicities requiring medication (RTOG Grade 2) were nausea (10/20 patients) and diarrhea (3/20). There were no treatment-related deaths.

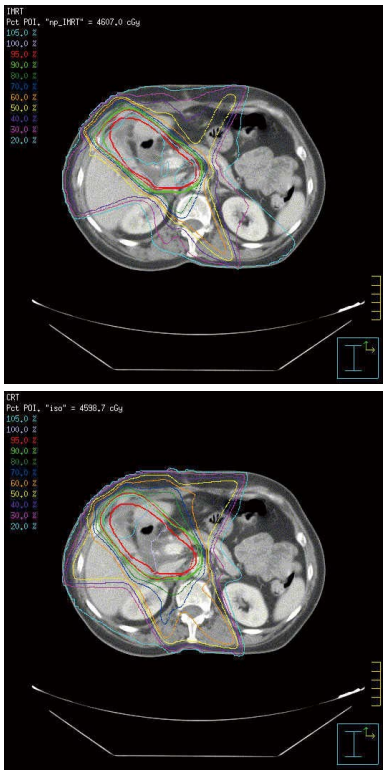

Figure 2 Isodose curves on an axial slice for a representative case. Intensity-modulated radiotherapy plan (upper picture) and conformal radiotherapy plan (lower picture).

Fourteen patients received chemotherapy (including concurrent chemotherapy), the major grade 3-4 adverse events were leukopenia (21%), neutropenia (29%) and anemia (14%). Compared with pre-treatment values, no abnormalities were detected in the laboratory test of kidney function for any of the 20 patients, either during treatment or at follow-up. Three patients had elevated liver enzymes about 6 mo after the completion of radiotherapy. No late toxicity was seen in this series.

Dosimetric comparison between 3D CRT and IMRT plans

To demonstrate the differences in dose distribution, Figure 2 shows isodose curves on an axial slice for one representative patient for IMRT and CRT. Figure 3 shows the DVH curves for the kidneys and liver at risk for one representative patient. Table 3 summarizes the mean doses to the PTV, kidneys and liver for CRT and IMRT plans. Compared with the CRT plan, IMRT significantly reduced the mean dose to the right kidney and liver, while the improvement in the dose to the left kidney was not significant. Both of CRT and IMRT limited the dose to spinal cord under 40 Gy. Table 4 summarizes the volume of critical structures receiving greater than the threshold dose[14]. Compared with CRT planning, IMRT significantly reduced the volume of right kidney receiving > 20 Gy and the volume of liver receiving > 30 Gy. IMRT has a

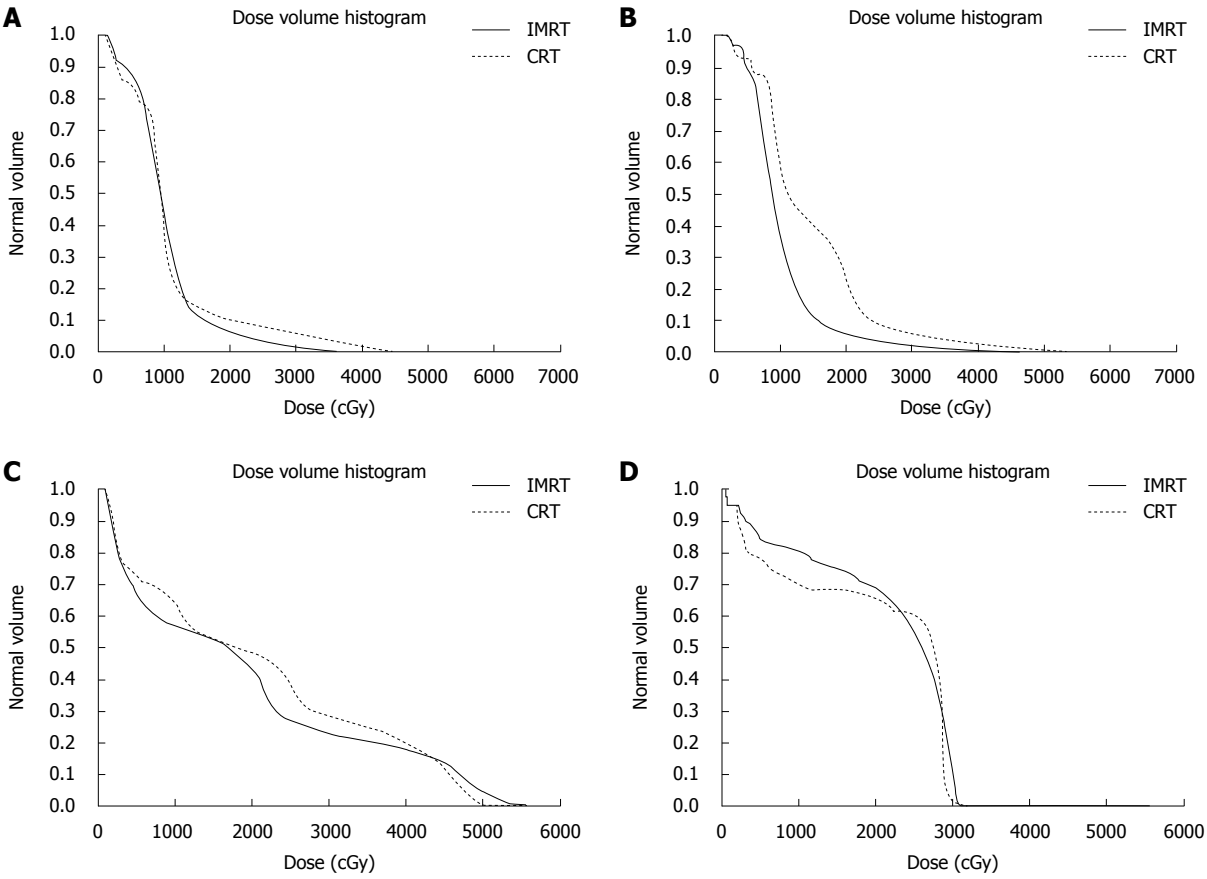

Figure 3 Dose volume histogram curves for the organs at risk in a representative case. Left kidney (A), right kidney (B), liver (C), and spinal cord (D). IMRT: Intensity-modulated radiation therapy; CRT: Conformal radiation therapy.

Table 3 Mean dose to the targeted structures/organs

Structure/organs	Mean dose (Gy)		*P* value[1]
	CRT	IMRT	
PTV	50.1 ± 2.8	51.5 ± 2.7	0.00015
Right kidney	10.6 ± 4.4	8.6 ± 2.4	0.032
Left kidney	8.1 ± 3.4	9.4 ± 4.6	NS
Liver	22.4 ± 3.9	21.0 ± 2.9	0.027

[1]Two-tailed paired *t* test. Values are expressed as mean ± SD. CRT: Three dimensional conformal radiation therapy; IMRT: Intensity-modulated radiation therapy; PTV: Planning tumor volume; NS: Not significant ($P > 0.05$).

Table 4 Volume of structures/organs receiving greater than the threshold dose

Structure/organs	Dose (Gy)	Volume above threshold dose (%)		*P* value[1]
		CRT	IMRT	
PTV	95% of prescribed dose	84.0 ± 6.7	82.9 ± 6.1	NS
Organs at risk				
Right kidney	20.0	12.5 ± 11.7	5.4 ± 2.7	0.031
Left kidney	20.0	5.3 ± 6.0	3.7 ± 2.3	NS
Liver	30.0	34.5 ± 8.0	30.1 ± 6.3	0.0065

[1]Two-tailed paired *t* test. Values are expressed as mean ± SD. CRT: Three dimensional conformal radiation therapy; IMRT: Intensity-modulated radiation therapy; PTV: Planning tumor volume; NS: Not significant ($P > 0.05$).

negligible impact on the volume of left kidney receiving > 20 Gy. The range of the 95% of prescribed dose for PTV using either 3DCRT or IMRT planning was 84.0% ± 6.7% and 82.9% ± 6.1%, respectively ($P > 0.05$).

DISCUSSION

Gallbladder cancer has a dismal prognosis and loco-regional recurrence has been described as the most frequent site of relapse, and death most commonly occurred due to complications and sequelae of loco-regional recurrence[15]. Hepatic infiltration has been reported in 60%-70% and nodal involvement in 20%-40% in some series[16,17]. The loco-regional pattern of recurrence after surgery provides a rationale for the use of radiotherapy as a component of gallbladder cancer treatment. The radiosensitive nature of gallbladder cancer is evidenced by numerous clinical studies reporting tumor size reduction after radiotherapy for unresectable diseases[18,19].

Our one-year survival rates and median preliminary survival time are similar to those reported in the limited radiotherapy literature and are generally better than those reported with surgery alone in patients with local advanced disease[20,21].

IMRT and automated optimization have the abil-

ity to shape isodose curves, avoiding the dose to the OARs. The use of direct machine parameter optimization IMRT improves the PTV coverage compared with CRT for most patients, although dose escalation was only possible in a minority of patients. It is suspected that the dosimetric improvement with IMRT was less in these plans compared with CRT plans. However, these complex CRT plans are challenging to develop without automated optimization, and IMRT might have planning efficiency and time-saving advantages for these cases.

Our data demonstrated that IMRT offers better sparing of the right kidney compared with CRT planning, with a significantly lower mean dose and volume above threshold dose. IMRT achieves similar excellent target coverage as compared with CRT, while reducing the mean liver dose and volume above threshold dose. In summary, IMRT offers improved sparing of normal structures, however, it warrants further studies in the treatment of gallbladder carcinoma.

In conclusion, gallbladder carcinoma is an aggressive disease with a dismal prognosis. More effective adjuvant therapy is needed to improve overall survival. There was a clear association between adjuvant therapy use and improved survival in patients with loco-regional disease. The real benefit of adjuvant radiotherapy in gallbladder carcinoma remains unclear. A retrospective analysis[21] was done about the surveillance, epidemiological, and end results survey by the American National Cancer Institute. The results showed that adjuvant radiotherapy is associated with improved survival in patients with locally advanced gallbladder cancer or gallbladder cancer with regional disease. Gallbladder cancer remains an aggressive disease that requires multimodality approach to individualize and optimize therapy. Prospective randomized trials of adjuvant therapy are needed in this disease. However, the low incidence of gallbladder cancer may make it difficult to successfully complete such trials, unless they are designed as inter-group studies within China or as international studies. In the future, methods of achieving earlier diagnoses may help improve the outcomes of the treatment. IMRT for dose escalation to improve tumor control and spare surrounding structure/organs from receiving radiation tolerance doses should be further studied.

COMMENTS

Background

Surgical therapy is the standard treatment for patients with resectable gallbladder cancer. Unfortunately, a large number of the patients develop recurrent disease despite curative resection. And local-regional failure is common and is a major cause of mortality. Because of this, adjuvant therapy has been used to improve loco-regional control and survival rate. Several studies have reported improvement for patients treated with adjuvant chemoradiation.

Research frontiers

Although gallbladder cancer is considered to be radiation resistant, radiation has been tried in the form of external beam radiotherapy, intra-operative radiation therapy and brachytherapy.

Innovations and breakthroughs

Intensity-modulated radiotherapy (IMRT) achieves similar excellent target coverage as compared with conformal radiotherapy (CRT) planning, while reducing the mean liver dose and volume above threshold dose. IMRT offers better sparing of the right kidney compared with CRT planning, with a significantly lower mean dose and volume above threshold dose.

Applications

The mainstay of treatment has been surgery and the role of adjuvant therapy in the form of chemotherapy and/or radiation therapy remains to be defined. Some clinical studies suggest that adjuvant radiotherapy dosage was associated with a better local control of the tumor. IMRT may offer better sparing of the right kidney and liver compared with CRT planning, this makes it possible for dose escalation to improve tumor control and spare surrounding structure/organs.

Terminology

IMRT: A type of three-dimensional radiation therapy that uses computer-generated images to match radiation to the size and shape of a tumor, which is used to deliver a higher radiation dose to a tumor with less damage to the nearby healthy tissues.

Peer review

The role of adjuvant radiotherapy in gallbladder cancer is not definite. This paper deals with a methodological comparison of CRT and IMRT for gallbladder cancer. IMRT offered better sparing of right kidney and liver compared with CRT. This result can help clinicians understand the role and toxicity of radiotherapy in gallblader cancer treatment.

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CASE REPORT

Hepatic veins as a site of clot formation following liver resection

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Abstract

Pulmonary embolism occurs more frequently after hepatectomy than previously thought but is infrequently associated with peripheral deep vein thrombosis. In this paper, we report 2 cases of postoperative hepatic vein thrombosis after liver resection. Both patients had undergone major hepatectomy of a non-cirrhotic liver largely exposing the middle hepatic vein. Clots were incidentally found in the middle hepatic vein 4 and 17 d after surgery despite routine systemic thrombo-prophylaxis with low molecular weight heparin. Coagulation of the transition plan in a context of mutation of the prothrombin gene and inflammation induced biloma were the likely predisposing conditions. Clots disappeared following curative anticoagulation. We conclude that thrombosis of hepatic veins may occur after liver resection and is a potential source of pulmonary embolism.

Key words: Hepatectomy; Hepatic veins; Thrombosis; Pulmonary embolism; Anticoagulants

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INTRODUCTION

Patients undergoing liver surgery have long been considered to be at low risk of venous thromboembolism. Routine Doppler ultrasound following major hepatectomies identifies deep vein thrombosis in 2% of patients[1], three to five times less than after general abdominal or colorectal procedures performed with adequate anticoagulation prophylaxis[2-5]. However, pulmonary embolism has recently emerged as an increasingly frequent and potentially fatal complication following liver resections. Its incidence ranges between 1% and 3% in patients undergoing liver resections[1,6] and has been reported to be as high as 10% in living-related donors undergoing a right hepatectomy[7,8]. These figures are greater than the 0.3% incidence observed following general surgery and the 2%-3% incidence observed after high risk

procedures such as invasive neurosurgery, total hip arthroplasty, and radical cystectomy[9]. Liver regeneration that follows major resections is indeed associated with an early and transient dysregulation of the haemostatic system resulting in a hypercoagulability state[10].

This difference between a low incidence of deep vein thrombosis and a high incidence of pulmonary embolism is difficult to explain as more than 90% of pulmonary emboli are considered to arise from lower extremity and pelvic deep veins[11]. Furthermore, less than 50% of patients developing a pulmonary embolism after liver resection have an associated deep vein thrombosis[7].

We shed a new light on this discrepancy by reporting two patients who developed thrombi in their hepatic veins following hepatectomy. To our knowledge, this complication has not been previously reported which can be explained by the technical difficulty to visualise the hepatic veins on imaging studies in the early postoperative period.

CASE REPORT

Case 1

A 39-year-old woman underwent a right hepatectomy for a 13 cm large liver hemangioma responsible for incapacitating pain. Hepatic veins were patent and besides a body mass index of 32 kg/m^2 she had no known risk factors for thromboembolic disease[12]. On the evening before surgery, tight-length graduated compression stockings were placed and she received a subcutaneous injection of 40 mg enoxaparin. Liver transection was performed using an ultrasonic dissector with two intermittent clamping of the hepatic pedicle of 11 and 15 min. Intrahepatic portal structures and hepatic veins were occluded with ligation, clips or bipolar coagulation as required. The right hepatic vein was closed extraparenchymally and the main trunk of the middle hepatic vein was retained with the left liver. Additional haemostasis of the transection surface was achieved with bipolar coagulation and the left liver was fixed to the diaphragm to prevent twisting of the hepatic veins under intraoperative ultrasound control[13]. No transfusion was required, the patient was extubated 3 h after surgery and daily administration of 40 mg enoxaparin was reinitiated on the following morning.

The early postoperative course was uneventful with rapid normalization of liver function tests but on the fourth postoperative day, she developed shortness of breath and a temperature rise at 37.8℃ at which time a computed tomography (CT) scan was performed. There was no obvious evidence of pulmonary embolism but three defects were found in the middle hepatic vein adjacent to the transection plan (Figure 1) that were confirmed to be 2-3 cm long clots by Doppler ultrasound. The inferior vena cava and termination of the middle hepatic vein had a normal flow pattern otherwise. Mild right pleural effusion, ascites and localised thrombosis of the right posterior tibial veins were also uncovered. Following administration of enoxaparin at 1.0 mg/kg twice daily, pulmonary symptoms disappeared within 48 h and control Doppler ultrasounds performed every other day showed the progressive disappearance of two of the three clots and the reduction in size of the third at which time she was discharged (postoperative day 21). The postoperative course had otherwise been uneventful. A control CT scan performed 1 mo later showed the complete disappearance of the clots. Screening for inherited thrombophilia identified a heterozygote (20210AG) mutation of the prothrombin gene while other risk factors, including factor V Leiden mutation were absent.

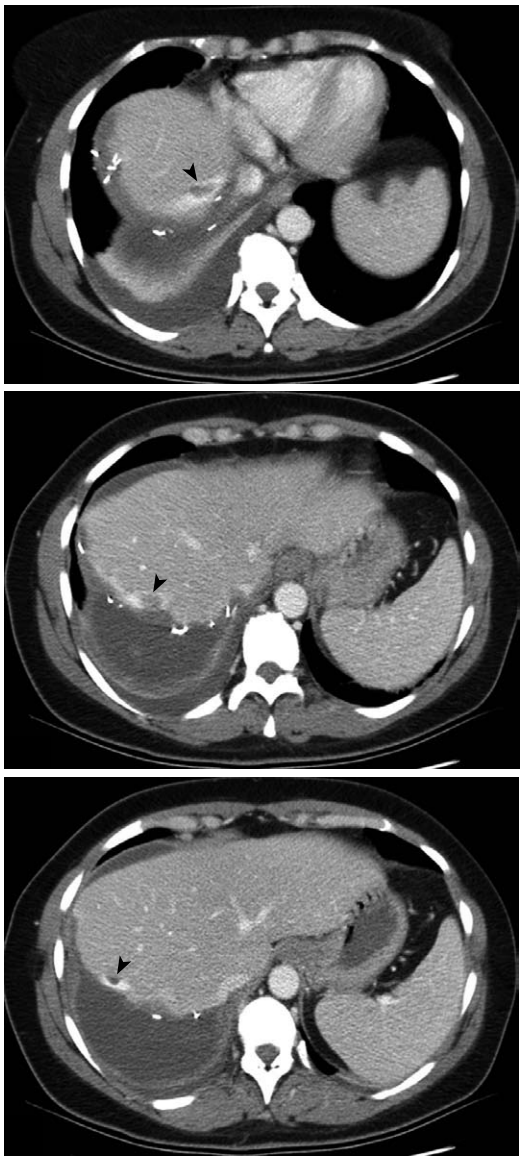

Figure 1 Postoperative computed tomography scan (day 4) in case 1. Defects are present in the middle hepatic vein close to the transection plan (arrowheads).

Case 2

A 78-year-old man underwent simultaneous left hepatectomy extended to a part of segment 8 by laparotomy and laparoscopic sigmoidectomy for synchronous colorectal liver metastasis. He had no history of thromboembolic disease and surgery was preceeded by 6 cycles of chemotherapy (FOLFOX regimen + cetuximab) that had induced a partial response according to RECIST criteria.

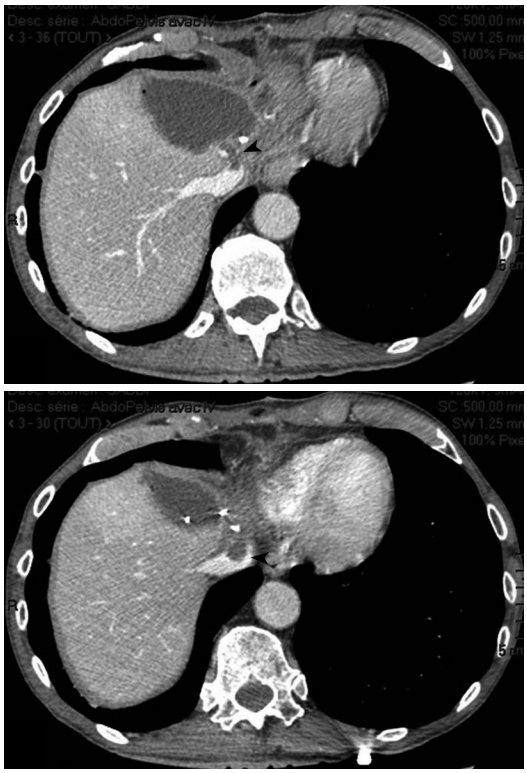

Figure 2 Postoperative computed tomography scan (day 17) in case 2. A biloma is present at the upper part of the transection plan and a defect is visible in the distal end of the middle hepatic vein extending in the inferior vena cava (arrowheads).

On the evening before surgery, tight-length graduated compression stockings were placed and he received a subcutaneous injection of 40 mg enoxaparin. Liver transection was performed as previously described, without vascular clamping. The middle hepatic vein was closed intraparenchymally while the left hepatic vein was closed extraparenchymally. No transfusion was required, the patient was extubated 3 h after surgery and daily administration of 40 mg enoxaparin was reinitiated on the following morning.

The early postoperative course was uneventful and the patient was discharged on postoperative day 11. He was re-admitted on postoperative day 17 for sepsis. CT scan showed bile collection close to the cut edge of the liver and a clot in the middle hepatic vein extending into the supra-hepatic vena cava (Figure 2). Percutaneous drainage of the collection was performed and anticoagulant therapy was administered, allowing complete regression of the thrombosis seven days later. Oral anticoagulation was then administered and the patient was discharged 10 d after admission (postoperative day 27). Screening for inherited thrombophilia was negative.

DISCUSSION

The present study reports two patients who developed early hepatic vein thrombosis following liver resection, a previously unrecognized complication. Both had several features in common: (1) they had undergone a major hepatectomy within a non-cirrhotic liver; (2) the thrombosis was located within the hepatic vein adjacent to the transection plan; and (3) was discovered somewhat fortuitously.

We have previously shown that major resections of non-cirrhotic livers are associated with an early postoperative decrease in coagulation inhibitors protein C and antithrombin together with an increase in factor Ⅷ and von Willebrand factor that induce a transient hypercoagulability state[10]. Both patients underwent a major resection while already being at increased risk of thrombosis. One indeed retrospectively proved to be overweight and to have a heterozygote (20210AG) mutation of the prothrombin gene associated with a three- and two-fold increase in the risk of thrombosis, respectively[14]. The other patient had an advanced malignancy that also increased the risk of thrombosis[12,14] and is associated with increased procoagulant activity[15].

The specific finding was, however, that thrombosis developed within the main trunk of a major hepatic vein adjacent to the transection plan. One patient (n°2) developed a biloma, an obvious local predisposing condition as local infection causes vein wall inflammation while general infection increases systemic procoagulant activity[16]. This was not the case in the other (patient 1) and we believe that thrombosis may have been favoured by extensive coagulation of the raw surface of the liver. Bipolar coagulation, which is widely used to achieve hemostasis during parenchymal transection, may result in heat-induced endothelial injury when a major hepatic vein lies close to the transection plan which is the case during formal right and left hepatectomies. Portal triad clamping, which is frequently performed during major hepatectomies, may also favour through stasis these coagulation-induced thrombosis[12].

The recognition of these thrombi in our patients was somewhat fortuitous which may explain why this complication might have previously been overlooked. CT imaging in the postoperative course of liver resections is generally indicated when a pulmonary embolism or an abdominal collection are suspected. Angio-CT scans are usually performed 15-20 s after the injection at which time hepatic veins are not visible. During more conventional CT scans, acquisition of images is similarly not always performed at the time hepatic veins are injected. Besides, the middle hepatic vein has a longitudinal direction and is therefore hardly visualized along a significant length by the transverse slices of CT scans.

Once present in major hepatic veins, these thrombi may easily migrate into the inferior vena cava as previously shown for tumour[17] or hepatic fragments[18]. Pulmonary embolism was not formally documented in our patients probably because anticoagulation therapy was initiated prior to clot migration.

In conclusion, thrombosis may occur in hepatic veins after liver resection as a result of intra- or postoperative local injury. This would explain why pulmonary emboli have been observed in the absence of peripheral deep vein thrombosis. This hazard should be taken into account when performing extensive coagulation of the raw surface of the liver when a major hepatic vein is exposed.

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Comments on the article about correlation between computerized tomography and surgery in acute pancreatitis

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Abstract

We read with great interest the article by Vege *et al* published in issue 34 of *World J Gastroenterol* 2010. The article evaluates the ability of contrast-enhanced computerized tomography (CECT) to characterize the nature of peripancreatic collections found at surgery. The results of their study indicate that most of the peripancreatic collections seen on CECT in patients with severe acute pancreatitis who require operative intervention contain necrotic tissue and CECT has a limited role in differentiating various types of collections. However, there are some points that need to be addressed, including data about the stage of acute pancreatitis in which CECT was done and the time span between CECT examination and surgery.

Key words: Acute pancreatitis; Pancreatic necrosis; Peripancreatic fluid collection; Contrast-enhanced computerized tomography; Surgery

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TO THE EDITOR

We read with great interest the article by Vege *et al*[1] published in issue 34 of *World J Gastroenterol* 2010. The article evaluates the ability of contrast-enhanced computerized tomography (CECT) to characterize the nature of peripancreatic collections found at surgery. For that purpose the authors excluded false positive and negative collections found on CT and presented their results in a comparative analysis. The results of their study indicate that most of the peripancreatic collections seen on CECT in patients with severe acute pancreatitis who require operative intervention contain necrotic tissue and CECT has a limited role in differentiating the different types of collections.

However, there are some points that need to be addressed. The authors neither specified in which stage of acute pancreatitis (pro-inflammatory or anti-inflammatory response) was CECT done nor they specified the time span between CECT examination and surgery. Since the clinical course of severe acute pancreatitis is very dynamic, and CECT and surgery were not performed concurrently, it may not be the matter of false negative and positive findings, but the collections could have rather be formed or disappeared in between CECT examination and surgery. Furthermore, the collections could have progressed from one stage to another, e.g. from necrotic to necrotic with pus or to liquefaction (as identified at surgery), which

could have also introduced significant bias into the analysis. We believe that this is a serious methodological limitation to this study which deserves attention, apart from having a significant number of unidentified collections with fluid but without necrosis on CECT.

By the way, the authors erroneously specified at the end of the 2nd paragraph in the Results section under the subheading *Peripancreatic collections* that 5 of 9 unidentified collections on CECT had associated necrosis and 4 had only fluid without necrosis, whereas it is obvious from Figure 1 that 4 collections had associated necrosis and 5 had no associated necrosis.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 04-05, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 01, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 03-05, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 07-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 06-07, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 07-09, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 07-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-02, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 02, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 01-04, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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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 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 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/index.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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