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TOPIC HIGHLIGHTS

Hans Gregersen, MD, Asbjorn Mohr Drewes, MD, *Series Editor*

Perception of stimuli in the gastrointestinal physiology and new methods: From the editor

This special issue of *World Journal of Gastroenterology* contains a number of reviews on physiology and perception of stimuli in the gastrointestinal tract. Pain is probably the most prevalent symptom in medicine and characterization of pain is of major importance in the diagnosis and choice of treatment. Consequently, understanding and characterization of the physiology behind gut pain is one of the most important issues in the diagnosis and assessment of organ dysfunction. Research leading to a better insight into pain mechanisms in the gastrointestinal tract will invariably improve the treatment of the patients. Experimental pain models have been used frequently in animal studies. However, animal experiments can only to some degree reflect the experience of clinical pain in humans. Because of these factors, experimental human pain models are advantageous to assess basic pain mechanisms and to investigate abnormal pain responses in functional and organic diseases of the GI system. Using these models, the investigator can control the experimentally induced pain and provide quantitative measures of the responses. Recently, techniques to assess the gastrointestinal anatomy and function have improved dramatically. Imaging with ultrasound combined with endoscope, functional luminal imaging and advanced modeling of the luminal structures with assessment of the mechanical properties of the tissues may be a new cornerstone in experimental and clinical gastroenterology. The key to the progress in these developments have been the interdisciplinary collaboration between doctors, biomedical engineers and mathematicians. In the future the new methods will undoubtedly lead us into a new area bridging the understanding of basic physiology, symptom assessment and daily clinical practice. The reader is referred to the papers in this issue of WJG for information on these important aspects of gastrointestinal function.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, Asbjorn Mohr Drewes, MD, *Series Editor*

Experimental human pain models in gastro-esophageal reflux disease and unexplained chest pain

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Abstract

Methods related to experimental human pain research aim at activating different nociceptors, evoke pain from different organs and activate specific pathways and mechanisms. The different possibilities for using mechanical, electrical, thermal and chemical methods in visceral pain research are discussed with emphasis of combinations (e.g., the multimodal approach). The methods have been used widely in assessment of pain mechanisms in the esophagus and have contributed to our understanding of the symptoms reported in these patients. Hence abnormal activation and plastic changes of central pain pathways seem to play a major role in the symptoms in some patients with gastro-esophageal reflux disease and in patients with functional chest pain of esophageal origin. These findings may lead to an alternative approach for treatment in patients that does not respond to conventional medical or surgical therapy.

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Key words: Pain; Gut; Experimental; Chest pain; Esophagitis

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INTRODUCTION

Pain is probably the most prevalent symptom in medicine and characterization of pain is of major importance in the diagnosis and choice of treatment[1]. Abdominal pain is frequently occurring, even in the normal population[2], and abdominal discomfort and pain are among the most common symptoms responsible for patients consulting the health care system[3]. Consequently, understanding and characterization of gut pain is an important issue in the diagnosis and assessment of organ dysfunction. Research leading to a better insight into pain mechanisms in the gastrointestinal (GI) tract will invariably improve the treatment of the patients[1]. However, in practice, assessment of clinical pain is difficult as the symptoms of the underlying diseases confound the description of pain. These confounders may include complaints relating to psychological, cognitive and social aspects of the illness, as well as systemic reactions such as fever and general malaise[4]. Treatment with analgesics may also cause sedation and other side effects. Finally, abdominal pain is much more diffuse than somatic pain with autonomic symptoms, referred pain and hyperalgesia in remote somatic tissues. These factors will invariable bias the clinical evaluation. For example in the evaluation of analgesics the patients often tend to interpret other effects of the medication-such as an effect on the anxiety and depression relating to the disease-as a relief of pain[5]. To encompass these bias experimental pain models are increasingly used, especially in animal studies. In these experiments, the neuronal nociceptive activity can be recorded or behavior can be assessed[6]. However, neuronal recordings or reactions do not reveal all aspects of pain being the net effect of complex multidimensional mechanisms that involve most parts of the central nervous system[7]. Furthermore, major species differences in pain mechanisms often influence the findings. Therefore, although nociceptive reflexes or electrophysiological recordings from selected pathways in the animal nervous system are important in basic research, the central pain mechanisms and associated complex reactions are typically suppressed. Thus, animal experiments can only to some degree reflect the experience of clinical pain in humans. Because of these factors, experimental human pain models are advantageous to assess basic pain mechanisms and to investigate abnormal pain responses in functional and organic diseases of the GI system. Methods related to

experimental human pain research aim to activate different nociceptors, evoke pain from different organs and activate specific pathways and mechanisms. Assessment of the output from these pain models can be based on psychophysical or neurophysiologic methods[8]. Using these models, the investigator can control the experimentally induced pain (including the nature, localization, intensity, frequency and duration of the stimulus), and provide quantitative measures of the psychophysical, behavioral, or neurophysiologic responses. Methods for imaging can also be added and some studies used a combination of these assessments. In this paper, however, the focus will be the methods for pain induction. The reader is referred to[4] for further detail regarding assessment of GI pain

EXPERIMENTAL PAIN MODELS

The ultimate goal of advanced human experimental pain research is to obtain a better understanding of the mechanisms involved in pain transduction, transmission, and perception under normal and pathophysiological conditions. Experimental approaches can be applied in the laboratory for basic studies and in the clinic to characterize patients with sensory dysfunction and/or pain in organic and functional diseases[4,9]. Depending on the experimental model, different central mechanisms and conditions mimicking pathological pain such as increased sensation to normal physiologic/non-painful and painful stimuli (allodynia and hyperalgesia) can be studied. However, most previous studies have relied on relatively simple mechanical or electrical stimuli. These methods are easy to apply but as discussed below they have special considerations and limitations. Therefore, new controlled and reproducible models and protocols are highly warranted.

The ideal experimental stimulus to elicit gut pain in man should be natural, minimally invasive, reliable in test-retest experiments and quantifiable. Preferably the pain should mimic the observations in diseased organs by evoking phenomena such as allodynia and hyperalgesia[10,11]. The different methods for pain stimulation of the human GI tract are described below and in Table 1. Some of the existing stimulation methods seem to fulfill the above requirements but most laboratories use their own stimulation paradigms, often without the necessary standardization.

Table 1 The different methods for pain stimulation of the human GI tract

Stimulation modality	Stimulated structures	Advantages	Limitations
Electrical	Nerve fibers primarily in mucosa and muscle layers dependent of the stimulation intensity (not a specific activation of the nociceptors)	Excellent for repeated stimulation, suitable for neurophysiologic assessments of the pain	The electrical threshold depends on the fiber diameter, i.e. small-diameter nerves cannot be excited without exciting others. May induce arrhythmias in areas near the heart
Mechanical	Mechanoreceptors located in different layers	Imitates a bolus, reproducible stimulus	Problems with estimating the transmural pressure and change in circumference
Thermal	Thermal sensitive receptors preferentially in the luminal layers	Activation of unmyelinated afferents in the mucosa selectively	Temperature stimuli in the range that can be felt are normally only relevant for sensation in the upper GI tract.
Chemical	Chemo-sensitive receptors, primarily in the mucosa	Resembles clinical inflammation, chemical stimuli activate predominantly unmyelinated C-fibres	Require a relative long latency time to the onset of effects, and that they are often not reproducible when repeated

Electrical stimulation

Depolarization of the nerve afferents by electrical current has been widely used as an experimental stimulus in humans for many years-for review see[9]. The electrical stimuli have proved to be safe in all parts of the gastrointestinal system[12-15].Various electrical stimulator devices connected to electrodes applied to the mucosa of the gut can evoke electrical stimulation. Stimulator devices can deliver different stimulation patterns, e.g., different waveforms, frequencies, and duration of the stimulus. This activates with some selectivity different afferents and nervous structures, and hence evokes different kinds of pain[7]. The electrical stimulus intensity to evoke pain as well as the size of the referred pain area is reliable and reproducible[12,13,16,17]. The well defined onset and offset of the stimulation eliminates the latency to stimulation of the afferents seen with other methods[7] and makes it suitable to study pain mechanisms related to time[16,18]. Thus, the method is very suitable for neurophysiologic assessments of the pain[18-20]. Furthermore, the stimulation field is usually not as large as in other models, and diseases with localized pathology such as ulcers may better be mimicked[13,18]. Central integration or temporal summation, being a human correlate to the initial phase of "wind-up", is a potent mechanism for generation of referred visceral pain and can easily be evoked by electrical stimulation[16,18]. The major shortcomings are that electrical stimulation bypasses the receptors and activates the nerve fibres directly, and the method is not a specific activation of the nociceptors. The electrical threshold is related to the fiber diameter and one cannot usually excite small-diameter nerves without additionally exciting others. Furthermore, electrical stimuli may induce arrhythmias when areas near the heart are stimulated[18,21] and therefore bipolar stimulation where the electrical field is more localized may be recommended.

Previous methods have suffered from problems with insufficient contact between the electrodes and the gut, and in some studies pain was not evoked in all

subjects. We have improved the method by integrating the electrodes on the biopsy forceps for the endoscopes, which makes it possible to stimulate well defined areas in the esophagus, stomach, duodenum, terminal ileum and colon[13,14,16,18]. Recently we used a 6 mm nasal endoscope for the stimulations. Apart from a little unpleasantness associated with the intubations, all subjects were able to lie comfortably and none complained of unpleasantness due to the endoscope for up to 4 h. An example of stimulation of the esophagus is shown in Figure 1. The advantage of this modification is that the position of the electrodes is controllable and can be changed in case of stimulation of nearby somatic structures and nerves. The contact with the organ wall is secured and evoked motor phenomena such as secondary contractions can be studied. However, endoscopic stimulation demands special equipment and facilities and improvement of methods for "blind stimulation" is highly warranted.

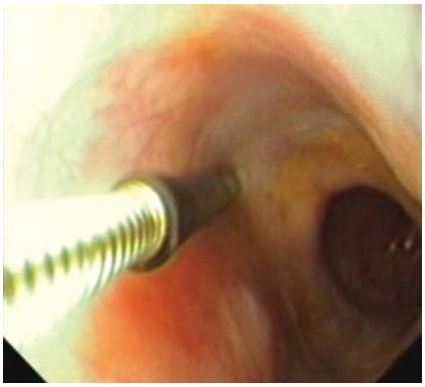


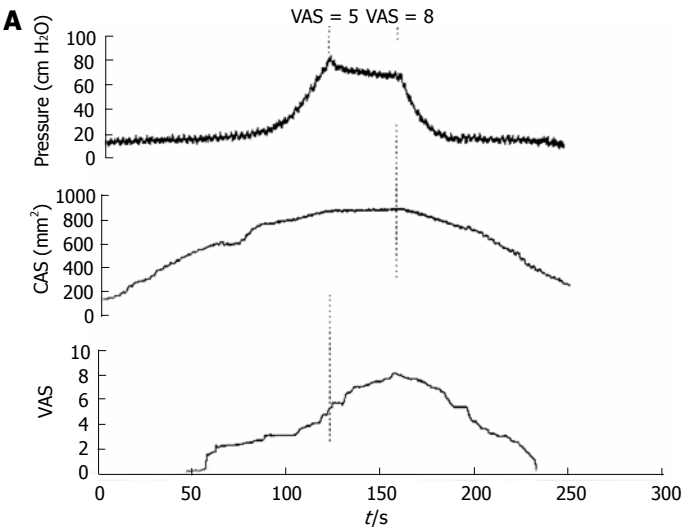
Figure 1 The stimulation electrode visualized in the distal esophagus using the nasal endoscope.

Mechanical stimulation

In the last decade several studies have addressed the mechanical and sensory function of the GI tract by means of mechanical distension. The mechanical properties of the GI tract are important for its function as a digestive organ and the gut contains mechanoreceptors at various locations in the wall, mainly in the muscle layers[6]. Mechanical GI tract stimulation, in particular balloon distension, has been widely used also to study GI smooth muscle tone[22-24], distensibility[22,25,26], peristaltic reflex responses[27], functional and organic disorders[28-32], referred pain and cerebral activation patterns[33] and the screening of new analgesics in healthy subjects and in patients with gut disorders[30,34-37]. There are several ways to stimulate the GI tract mechanically. Simple and physiological methods for distension of the gut such as ingestion of well-defined meals may be useful in clinical studies[38]. Balloon distension is, however, much more used than ingestion as mechanical stimulation method and easier to control. Therefore, the following sections will focus on controlled balloon distension methods used to evoke non-painful sensations and pain in the gut.

Most recent studies have used a method of measuring changes in volume of air in a balloon at constant pressure levels named "the Barostat". Several protocols and stimulation paradigms have been recommended for the Barostat, such as for example "phasic and tonic distensions". The stimulation paradigms have been thoroughly discussed recently and will not be described here-for review see[30,39]. The great advantage of the Barostat system and similar pressure-volume based methods are the relatively low cost, especially if the system is self-made. Furthermore, they are easy to use for routine purposes. Such systems have been used for determination of sensory and pain thresholds, and under different conditions attempts have been done to calculate the compliance and tension of the organs[26,30,39,40]. However, there are drawbacks with these simple methods. For example using the Barostat device, the data must be corrected for compressibility of air. The four major concerns, however, relate to (1) elongation of the balloon during distension; (2) the use of very long balloons; (3) the use of erroneous assumptions; and (4) the improper use of distension protocols. In more detail: (1) Balloon distension in a tube will cause the balloon to elongate to some degree due to the resistance to deformation in radial direction[41]. Volume is therefore not an accurate measure for the distension of the organ; (2) The use of long balloons is problematic because phasic contractions can be misinterpreted as muscle tone[42]. In addition relaxation in one end of the balloon and simultaneous contraction in the other end may not be registered as a change in volume at all; (3) Most previous studies have not validated the assumptions the data were based on. The most obvious and common mistake is to consider the fundus of the stomach to be spherical. From an anatomical and geometric point of view the stomach is not spherical and the wall structure with muscle layers in various directions indicates complex (anisotropic) properties. Distending a balloon in the fundus will deform the balloon into the corpus and antrum, resulting in a highly complex geometry; (4) No previous pressure-volume studies have considered the strain softening effect[43] and therefore it is highly questionable whether the results are reproducible. The tissue needs to be preconditioned before proper testing

The first three of the points mentioned above makes it impossible to compute the radius from the measured volumes. Consequently, estimation of wall tension using Laplace's law and computation of deformation in terms of strain measures will be invalid. It has also been a common mistake in gastrointestinal distension studies to consider the mechanoreceptors to be pressure receptors, volume receptors or tension receptors. Circumferential strain or stress are more likely candidates as the direct receptor stimulus, because the tensile stress and strain in distensible biological tubes are largest in the circumferential direction during distension[44]. Considering mechanical and receptor kinematic properties, strain is a more relevant parameter than stress (and tension). This is partly due to the fact that strain is a non-dimensional parameter that is independent of the original size of the organ and directly associated with tissue deformation. Recent studies clearly demonstrated that circumferential strain is an important determinant of mechanoreceptor mediated responses[45-48]. Correspondingly, studies providing tension calculations from Barostat studies have shown conflicting results, e.g.



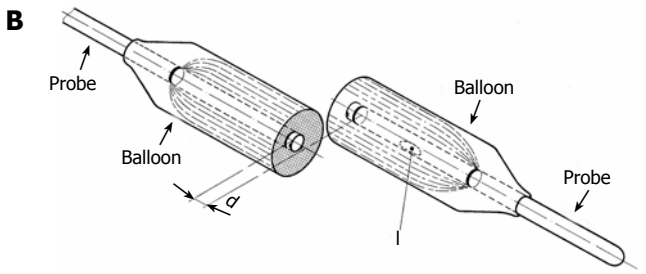


Figure 2 Top corner: The impedance planimetric probe for mechanical stimulation of the gastrointestinal tract. l and d denote infusion side for filling the balloon and the distance between two detection electrodes. Left: Recordings in the small intestine using the impedance planimetric probe with pressure, cross-sectional area (CSA) and VAS response as function of time during a ramp distension of the balloon up to the pain threshold where the bag volume is kept constant. It appears that the pressure decreased, the CSA was constant whereas the pain continued to increase.

in a recent study of the stomach the estimated tension seemed to correlate with the sensation[26,49], whereas another study[35] showed a highly inter-individual variability in the sensation score to the applied tension, suggesting that other factors than wall tension influence the sensation. However, uncertainties in the assumptions given above, and lack of proper geometric and biomechanical considerations can also explain these findings[41].

Newer methods based on impedance planimetry (Figure 2) allows recording of the luminal cross-sectional area directly and estimation of the radius in the distended segment of the esophagus or intestine[17,45,47,48,50,51]. From the radius it is possible to compute the circumferential wall tension and strain with more accuracy than from volume measurements[44]. Circularity must be assumed but this assumption has often been validated[44]. Finally, mechanical distension combined with ultrasound methods may offer the possibility for a better anatomical characterization and computation of stress in the GI tract[52]. For the stomach and other organs with complex geometry, imaging methods that provides data on the surface curvatures (3D-ultrasound, multislice CT-scanning or MR-scanning) in the whole organ may be used together with numerical mechanical analysis such as finite element analysis.

Thermal stimulation

In the skin rapid heating activates first myelinated Aδ-fiber, where the evoked sensation corresponds to the "first pain felt within 0.4 s after the heat stimulus. The first pain is followed by a C-fiber mediated second pain which is less well localized and of longer duration, being described as 'throbbing, burning or swelling'[53]. Slow heating gives a preferential activation of the unmyelinated C-fibers and the best evaluation of second pain[54]. Short lasting thermal stimuli have also been used in the human GI tract. Here they are believed to activate unmyelinated afferents in the mucosa selectively as opposed to mechanical and electrical stimuli, which activate afferents in both the superficial and deeper layers[6]. Thermosensitive mucosal afferents throughout the gut have been demonstrated in animal studies[6], and in the human esophagus, stomach and rectum[6,17,55-57]. The major limitation of these models is that temperature stimuli in the range that can be felt are normally only relevant for sensation in the upper GI tract. On the other hand, although temperature receptors may have less clinical relevance, characterization of the response to stimuli in the most superficial layers of the gut may be of major importance to develop a comprehensive sensory evaluation[4]. In recent studies the temperature of re-circulating water was continuously measured inside a balloon positioned in the esophagus[17,58]. The temperature stimuli showed a nearly linear stimulus-response relationship, demonstrating the validity of the receptor activation.

Chemical stimulation

Chemical stimulation of the GI tract more closely resembles clinical inflammation and is believed to approach the ideal experimental visceral pain stimulus[9]. Such stimuli have successfully been applied to the skin and muscles[11,59,60]. In contrast to electrical stimuli, chemical stimuli activate predominantly unmyelinated C-fibres[61] and this may be an advantage in basic studies of differentiated nerve populations. Acid stimulation of the esophagus is the most used method to sensitise the gut[59,62-65] but chemical stimulation of the gut with alcohol, bradykinin, glycerol, capsaicin and hypertonic saline were also done in humans[18,55,58,66-69]. For details see[4]. The major disadvantage of chemical stimuli is that they require a relative long latency time to the onset of effects, and that they are often not reproducible when repeated[9]. Thus, although chemical stimulation may evoke pain, the major relevance of the model is in sensitization of the visceral afferents to subsequent experimental stimulation.

Ischemic stimulation

Ischemia has been widely used for induction of muscle pain. Ischemia is important in many diseases such as mesenteric ischemia and ischemic colitis. Ischemia has been used in animal models of visceral pain[9]. Such studies are not acceptable in humans and unfortunately no human experimental gut models exist. However, distension of the gut may result in diminished blood flow. Accordingly, Öhman *et al*[70] observed a significant drop in blood supply during distension of balloons in the small intestine in rats.

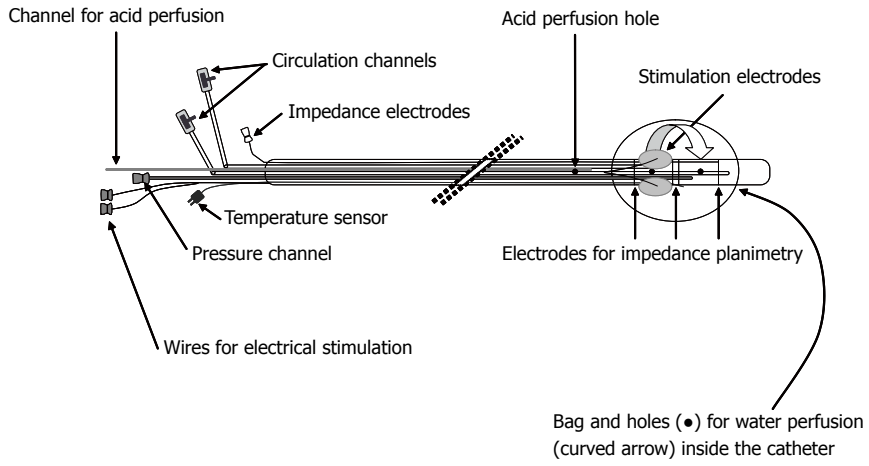


Figure 3 Schematic illustration of the probe used for multi-modal (electrical, mechanical, cold and warmth stimuli) of the esophagus. The stimulations can be combined with sensitization using acid perfusion of the distal esophagus.

Very recently, Hoff and coworkers presented a multimodal probe that selectively was developed for differentiation between mechanical and ischemic pain mechanism[71].

Multimodal stimulation of the GI tract

As pain is a multidimensional perception it is obvious that the reaction to a single stimulus of a given modality can represent only a limited fraction of the entire pain experience. The possibility for combining different methods for stimulation and assessment will approximate the clinical situation where many different nerves and pathways are activated. Thus, the method will give a more comprehensive and differentiated information about the nociceptive system compared to stimuli using single or a few modalities[72]. For comprehensive experimental studies mimicking the clinical situation, a multimodal testing approach must therefore be used, where the test battery will increase the probability for activation of a range of relevant nervous mechanisms. Such sophisticated methods will be able to select the best test procedures to explore different basic aspects of pain as well as pharmacological modulations. Multimodal models have shown their value in somatic pain models, where single modality models have been inadequate to test for example pathophysiological changes and effects of specific drugs[73,74]. Especially if the stimulation includes modalities known to evoke peripheral as well as central sensitization, the likelihood that a model will mimic clinical pain is high despite the non-harmful nature of the stimulation[73,75]. In somatic models it is possible to give many different stimuli and evoke central phenomena (e.g. central integration of summated stimuli) of clinical relevance (for review see[11,59]). In the GI tract, however, difficulties with access to the organs and technical limitations of the currently available models have until now made such a multimodal stimulation approach difficult. Some authors have combined mechanical and electrical stimuli[12,75,76] or mechanical and chemical stimuli[77,78], and in a recent study in the esophagus mechanical and electrical stimuli were combined with sensitization to acid[79]. We recently introduced a multimodal model with combined mechanical, electrical, cold and warmth stimuli of the esophagus together with acid sensitization[17,58] (Figure 3). The multimodal approach gives the possibility for a differentiated stimulation of receptors in the superficial and deep layers of the gut. The possibility for induction of hyperalgesia with e.g., acid perfusion and evoking central phenomena such as summation, allodynia and referred pain makes the model clinically relevant with respect to increase the knowledge of peripheral and central pain mechanisms[64,65,80]. The pain assessment should ideally also be multimodal and for example include quantitative and qualitative sensation, assessment of referred pain and neurophysiologic measurements. For details see[4]. However, is will typically not be feasible with too many stimulation and assessment parameters.

METHODS FOR AMPLIFICATION OF THE CENTRAL NEURONAL RESPONSE

Chronic pain is characterized by modifications of the central nervous system such as central sensitization[81]. Hence, decreased thresholds to normal stimuli such as stools and air in the GI tract seem to contribute to many of the symptoms reported by patients with inflammatory and functional diseases in the gut[82,83]. Phenomena relating to sensitization of the central nervous system (CNS) are enhanced responses to repeated stimulation, allodynia/hyperalgesia and increase in the referred pain area[4]. Repeated electrical or mechanical stimuli to the small and large intestine[16,84,85] may cause integration of the neuronal response and thus pain, and this may be used as a model for enhanced central gain (Figure 4). Munakata *et al* showed the importance of central mechanisms in functional gut disorders[86]. In their study the patients developed rectal hyperalgesia following repetitive sigmoid distensions. Induction of hyperalgesia in one area of the GI tract following conditioning of the more proximal parts most probably results from central sensitization. Chemical stimulation may also evoke sensitization. Acid stimulation of the esophagus has been used as a chemogenic stimulus by several authors[76-79,87]. Increased response to mechanical, electrical and thermal stimuli after the chemical sensitization have been demonstrated[58,76,78,79], although previous studies using latex balloons were not as consistent. This may be related to methodological problems using latex balloons, where the distension data need to be corrected for the balloons intrinsic mechanical properties *etc*.[44,78]. Sarkar *et al*[79] demonstrated the relevance

of central mechanisms in the gut, as acid exposed to the esophagus not only resulted in local allodynia, but also sensitized the proximal, non-acid exposed part of the organ to electrical stimuli. Capsaicin also increases the sensation in the esophagus to acid reflux[88,89] and when capsaicin was applied to the small intestine, consistent hyperalgesia to mechanical stimuli was seen[18]. Lipid infusion in the duodenum has also been shown to induce allodynia and increase the referred pain area to distension in functional disorders[90,91]. As fat is a natural stimulus to the gut, such studies may increase the understanding of the postprandial symptoms in these patients.

EXPERIMENTAL PAIN AND GASTRO-ESOPHAGEAL REFLUX DISEASE

Gastro-esophageal reflux disease (GERD) is defined as chronic mucosal damage or typical symptoms, which reduce quality of life by the abnormal reflux of gastric contents into the esophagus. GERD is very common in the population with up to 30% of the European population reporting heartburn and/or acid regurgitation during the previous 12 mo[92]. Recent studies revealed that up to 70% of the GERD patients have non-erosive reflux disease (NERD) according to endoscopy, 24-h pH profile and symptom-reflux association indices. However, in patients with NERD, the quality of life impairment is comparable to that in patients with erosive esophagitis[93]. The symptoms in reflux disease are highly variable and poorly understood. Thus, in patients with GERD no simple relation seems to exist between the symptoms and severity of the disease[94-96]. Although treatment with proton pump inhibitors (PPI) is very effective, many patients continue to have symptoms despite treatment[97,98], and in a recent study 50% of the patients continue to have pathologic reflux despite effective symptom control with PPI[99]. Furthermore, it is estimated that 30%-60% of patients with NERD will have normal ambulatory 24-h esophageal pH monitoring and thus no effect of PPI treatment[93]. Although some of these patients may have reflux of non-acid gastric contents, it is still not clear what causes the symptoms in many patients.

Experimental pain methods have contributed to the understanding of the symptoms in reflux disease. In an animal study Garrison *et al*[100] demonstrated that spinal neurons in the cat receiving input from the distal esophagus also received convergent input from the thoracic wall and the heart. When the esophagus was sensitized with turpentine, the neurons responded to a smaller mechanical stimulus from the different sites. Such data gives evidence that central mechanisms may explain the symptoms in a substantial proportion of the patients. In humans, however, relative few studies have been done to explore the pain mechanisms in reflux disease.

Non-erosive reflux disease

Peripheral pain mechanisms: Assessment of mechanosensitivity using intra-esophageal balloon distension has yielded contradictory results. In NERD patients Rodriguez-Stanley *et al*[101] reported a decrease in sensation and pain thresholds to distension compared with a historic control group. Trimble *et al*[102] studied NERD patients without excessive reflux and found that these patients were most sensitive to esophageal balloon distension, whereas patients with excessive reflux had a level of sensitivity similar to that of healthy control subjects. In another study using esophageal balloon distension delivered by an electronic barostat, patients with NERD and patients with erosive esophagitis did not demonstrate an increase in mechanosensitivity when compared to normal controls[87]. The previous experimental pain studies used mechanical stimulations based on recording of volume and pressure. As stated above these studies may lead to errors relating to the deformation field and erroneous conclusions[47]. Is has been suggested that chronic esophageal exposure to excess acid affects chemosensitive but not mechanosensitive afferent pathways[87] and that the key abnormality in NERD patients is that they are hypersensitive to acid reflux. Recently we conducted a study in patients with NERD where 50% had a positive 24-h pH measurement. Furthermore a multimodal approach was used. Our data showed that the patients had hyperalgesia to heat stimulation, whereas they were hyposensitive to mechanical stimulations. There was a difference in the NERD subgroups as patients with a pathological pH profile exhibited hyposensitivity to mechanical stimulations compared to both controls and patients with normal pH monitoring (Figure 5). Taken together with the above findings the results reflect that patients with pathological acid reflux may be less sensitive to mechanical stimulation and more sensitive to heat. The selective sensitization to heat may be related to specific receptor activation. Recently, we showed that acid perfusion of the esophagus in healthy subjects differentially sensitizes the esophagus to heat, but not cold stimuli[80]. The VRI receptor may be important for our findings. The receptor can be activated by a variety of stimuli, including acid (protons) and increases in temperature that reach the noxious range. Thus, it can be hypothesized that in patients with NERD the acid reflux and resulting peripheral sensitization

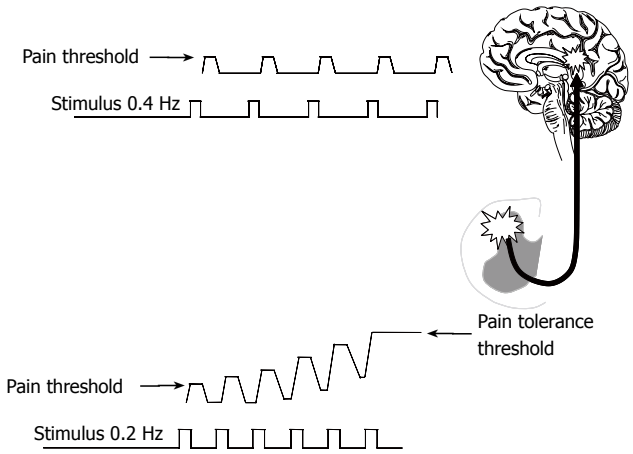


Figure 4 Central integration of repeated stimulation is seen when the stimulation frequency exceeds 0.3 Hz (bottom picture), whereas slower stimulation does not change the central gain (top picture).

results in a significant change in the sensation to heat stimuli working on the same receptor as the acid. Further studies need to explore whether the VRI receptors are up-regulated as seen in patients with erosive disease of the esophagus[103]-see below.

Central pain mechanisms: In the studies described above we also found an increase in the referred pain areas for both mechanical and heat stimulation in NERD patients. Previously we showed that acid perfusion of the distal esophagus in healthy subjects resulted in an increase in the referred pain area to differentiated esophageal stimuli[58]. This is most likely related to central neuronal hyperexcitability after acid perfusion, and subsequent opening of latent connections between converging neurons from visceral and somatic structures in the CNS[81]. Thus, the larger and widespread localization of the referred pain area is thought to represent central hyperexcitability. Consistent with these findings Penagini *et al*[104] showed that patients with NERD had increased sensitivity to distension of the proximal stomach. This viscero-visceral hyperalgesia is also considered a central phenomenon[105]. Experimental acid perfusion of the esophagus in healthy subjects has also been shown to increase the amplitude of the polysynaptic nociceptive reflex working at the spinal level[58]. Sarkar *et al*[79,106] demonstrated that acid perfusion of the distal esophagus resulted in allodynia and shorter latencies of the evoked brain potentials to electrical stimulation of a more proximal segment of the esophagus. Accordingly, we recently found a backward shift of the early activity in the cingulate gyrus to esophageal pain stimuli after acid perfusion of the organ[107]. Thus, there is substantial evidence that exposure of acid in the esophagus such as in some NERD patients may result in central neuroplastic changes at spinal and supraspinal levels.

Erosive GERD

Peripheral pain mechanisms: Patients with erosive disease of the esophagus may have a more severe disease than NERD patients, although erosive disease may also be a distinct entity[108]. In a study comparing patients with esophagitis with controls, Fass *et al*[87] demonstrated enhanced perception to acid perfusion, but the response to mechanical stimulation was normal. Such findings may point towards a differential effect on mechano-sensitive and chemo-sensitive pathways in esophagitis. Recently, we compared the sensory response in patients with grade B esophagitis and healthy controls using the multimodal approach described above. The patients had hyposensitivity to the mechanical stimulations, but had hyperalgesia to heat. These findings were comparable to those in NERD patients with abnormal pH profile and indicate that the pain mechanisms may be the same whenever erosions occur or not. A recent paper suggested that abnormal tissue resistance to acid may explain both the hyperalgesia and motor abnormalities seen in many patients with GERD and NERD[109]. In our studies the patients showed hyperalgesia to heat (but not cold) stimuli[110]. We believe that VR1 receptors sensitized by the acid reflux are important, and VR1 receptors have recently been shown to be up-regulated in esophagitis[103].

Central pain mechanisms: Central changes are also

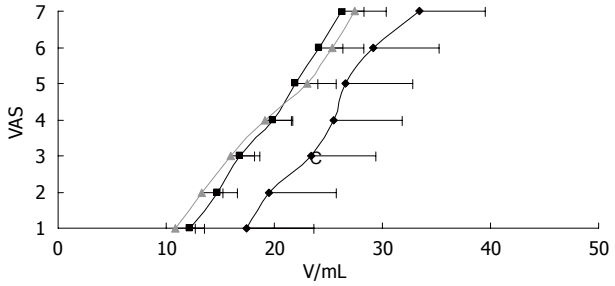


Figure 5 Stimulus-response function (mean ± SEM) to mechanical stimulation in patients with non-erosive gastro-esophageal reflux disease (NERD). The controls (■, *n* = 15) and patients with a normal 24-h pH-profile (▲, *n* = 7) had similar response to the mechanical stimulation, whereas patients with pathological pH-profile (◊, *n* = 6) showed hypoalgesia to the stimulations. The x-axis shows the volume of the balloon in the distal esophagus and the y-axis denotes the pain response on a visual analogue scale (VAS) with 5 as the pain threshold.

believed to be important in erosive disease. Although Fass *et al*[87] found a normal location of the referred pain we recently showed that the size of the referred pain area was larger than in controls. Thus, there is substantial evidence that exposition of acid in the esophagus in patients with esophagitis results in central neuroplastic changes. One can speculate that the reason for specific hyposensitivity to mechanical stimulations in patients with erosive disease may be related to well functioning counter-regulatory neural mechanisms acting from the brain stem at the spinal cord level. These may prevent the development of long-lasting sensitization of mechanosenstive afferent pathways[111]. The central pain modulating systems rely on a balance between facilitatory and inhibitory descending pathways and intrinsic spinal circuits and is not predictable in the individual patient[112,113]. Studies on animals have shown that the system is an important mechanism in the modulation of visceral stimuli[114] and these neuroplastic changes may result in increased referred pain on the one hand and dampening of the activity from mechanosensitive pathways on the other. In general chronic tissue injury and pain has been associated with higher thresholds to mechanical stimulation in different regions of the gastrointestinal tract. For example, chronic inflammation of the small bowel[115] in patients with inflammatory bowel disease is not associated with mechanical hyperalgesia of the rectum. This is contrasted by the pain in functional visceral disorders where hyperalgesia and allodynia to mechanical stimuli of the gut are typically found[86,115-118]. Hence, it can be speculated that a difference in the balance between noxious control systems arising in the brainstem may explain the findings in the different patient groups.

Non-cardiac chest pain

Non-cardiac chest pain (NCCP) was a term invented by the cardiologists. It was defined as "angina-like" chest pain without demonstrable abnormalities in the coronary vasculature. The more broad term "unexplained esophageal chest pain" is probably a better definition in gastroenterology, and may be defined as chronic chest pain, which is most likely of esophageal origin, but where

conventional tests for cardiac, esophageal, pulmonary (pain can be evoked from the pleura only) and musculoskeletal disorders are negative. The pain can be angina-like, but can also mimic other chest pain disorders such as gastro-esophageal reflux disease and musculoskeletal disorders. However, in the following the term NCCP will be used as it is the most common description of the syndrome. NCCP is very common especially in the younger age groups and the prevalence ranged from 22%-30% in the general population[119,120]. However, many persons have only modest and intermittent complaints and such cases must be classified as intermittent chest pain that is of no major burden, and a rough estimate would be that severe symptoms are seen in 2%-3% of the population.

If the patients describe "heartburn" and "acid regurgitation" they are typically classified as having GERD. However, burning pain may also be evoked by e.g., experimentally distension or electrical stimulation of the stomach[121] and the value of the subjective description of chest pain is up to discussion. GERD is also the most frequent cause of NCCP[122,123]. In this case the chest pain is no longer unexplained and the overlap invalids the diagnostic criteria for NCCP. The reason for heartburn in GERD is also up to discussion. Fass *et al*, have demonstrated that acid is not the only visceral stimulus that leads to heartburn[87], and recently Balaban *et al*, suggested that longitudinal muscle contractions of the esophagus as detected by high frequency intraluminal ultrasound, may be the motor equivalent of heartburn sensation[124]. Thus there is major overlap between the symptoms relating to the different chest structures.

The origin of chest pain

The symptom description is consistent with the organization of the nerves supplying the esophagus and the chest. The visceral afferents converge on a large scale with neurons that receive input from superficial and deep somatic tissue as well as other viscera[105]. Accordingly, in the thoracic spinal cord more that 75% of all neurons receive both somatic and visceral information, being contrasted by the actual number of visceral afferents (5%-15% of the inflow) entering the dorsal roots[6,125]. The visceral afferent terminals are widely distributed in the spinal cord and some organs have a wide spinal representation spreading to several segmental levels. For example the esophagus is innervated craniocaudally from the mid-cervical to the second lumbar segments[6]. Therefore a few visceral afferent fibers can activate many central neurons. Through the extensive functional convergence of somatic and visceral nerves on the same central neurons this wide activation of the CNS may explain the diffuse and unpleasant nature of visceral pain together with referred pain to somatic structures and other viscera[126]. In the clinic the above considerations typically leads to a very diffuse and heterogeneous description of the symptoms and experimental pain models may shed light over the pathogenesis of NCCP and related symptoms.

Peripheral pain mechanisms

Experimental pain studies in patients as outlined in the Introduction section have the possibility to control the stimulus and assessment parameters, and thus explore the abnormal pain system in further detail. Most studies found that the sensation and pain detection thresholds to distension, electrical and acid stimuli of the esophagus were lower in NCCP patients compared with thresholds found in healthy subjects[76,79,127-132]. However, the reliability of the test for diagnosing NCCP has been questioned[4], and in some experiments only few patients had abnormal sensation[133]. There are, however, severe methodological problems relating to these studies. Early balloon distension studies were based on simple volume and pressure measurements using latex balloons. As stated in the section above "Mechanical stimulations" this caused large errors due to the deformability of latex and lack of control of the stimulation field. There are also limitations and sources of error with the systems based on volume and pressure. The use of methods based on impedance planimetry has encompassed many of these limitations. Rao *et al*[134] used impedance planimetry and showed that patients with NCCP suffered from esophageal hyperalgesia. Furthermore the esophagus was more "irritable" than in controls, as the distensions resulted in more vigorous contractions at lower pressures. In more recent studies hyperalgesia was also found after relaxation of the smooth muscle with atropin[135], and there was evidence that the lower segments of the esophagus exhibited a higher level of sensory dysfunction compared to higher segments[136]. These studies were based on phasic distensions, which did not take the strain softening effect into account. Hence, we recommend to preconditioning the tissue by several distensions until the stress-strain relationship becomes reproducible[17,45,47].

Central pain mechanisms

Paterson *et al*[137] showed that repeated distensions conditioned the esophagus in NCCP resulting in higher pain scores. The effect of repeating stimulations has also been demonstrated in patients with irritable bowel syndrome, and probably this is a phenomenon relating to central hyperexcitability[86]. In a recent study we used a ramp distension protocol to distend the distal esophagus in patients with NCCP before and after sensitization with acid[138]. Patients with NCCP did not seem to be more vulnerable to develop esophageal hyperalgesia to the slowly increasing mechanical distensions as compared to controls. However, there was evidence for abnormal central pain processing as there was an increased and widespread referred pain area to the mechanical stimulations (Figure 6) and the patients were sensitive to repeated mechanical stimulation. Furthermore, after acid perfusion (believed to evoke hyperexcitability of central pathways mainly) there was a major sensitization to the distensions. Thus, it was concluded that NCCP patients showed facilitated central pain mechanisms, which may explain the character of their symptoms. This may have important implications for the diagnosis and treatment in these patients, where drugs with central effects should be used in the treatment.

Several studies have focused on brain activation to experimental pain stimuli in NCCP. The

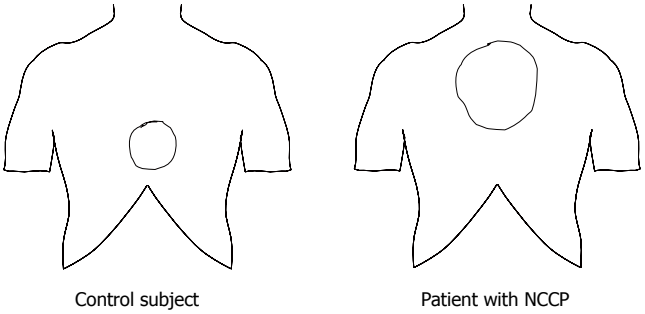


Figure 6 The localization and size of the referred pain area to mechanical stimulations of the distal esophagus in a typical control subject and a patient with non-cardiac chest pain (NCCP). The patient had and increased size and abnormal localization of the referred pain.

electroencephalography monitors the brain activity to external stimuli directly in real time. When a repetitive stimulus is applied and the cortical electrical activity is averaged (time-locked to the stimulus), the stimulus evoked cortical potential (EP) can be extracted from the background electrical activity and is shown in shape of a waveform with different peaks. Each peak in the EP represents a synaptic event associated with the transmission of afferent information from one group of neurons to another. The early peaks are supposed only to be influenced by the stimulation rate, intensity and localization, and they reflect to a major degree the brain loci that process the pain intensity and localization[139,140]. Three studies showed lower EP amplitudes to esophageal electrical and distension stimuli in patients with NCCP compared with healthy subjects[127-129]. Furthermore, Hollerbach *et al*[128] found shorter latencies of the EP peaks to electrical esophageal stimulation and more pronounced changes of the outflow of the autonomic nervous system in NCCP patients. Frobert *et al*[141] stimulated additionally the sternal skin (referred pain area) and showed shorter latencies and lower amplitudes of the EP to skin stimulation in NCCP patients. These findings suggest an increased central nervous system response to visceral stimuli in patients with NCCP comparable to those found in other groups with functional bowel disorders, e.g. patients with irritable bowel syndrome. Recently, Hobson and Aziz [142] suggested that different subgroups of patients with NCCP may exist: (1) Those with short latency of the early EP components having sensitization of gastrointestinal afferent pathways, and (2) those with long latencies and enhanced late responses reflecting hypervigilance and increased affective processing.

CONCLUSION

Pain is the most prevalent symptom in gastroenterology but yet poorly understood. This is reflected in the treatment of visceral pain that is often very difficult and highly challenging. Methods to evoke and assess experimental pain in the GI tract have recently been improved and used to explain the pain mechanisms in health and disease. In diseases relating to the esophagus these methods have contributed to our understanding of the symptoms reported in these patients. Especially does abnormal activation and plastic changes of central pain pathways seem to play a major role. These findings may lead to an alternative approach for treatment in patients that does not respond to conventional medical or surgical therapy, and probably analgesics with effect on the central nervous system such as tricyclic antidepressives, serotonin reuptake inhibitors, ion-channel blockers or opioids with additional effect on the N-methyle-D-aspartate (NMDA) receptor or kappa- opioids receptors should be used. Phase II trials where the experimental methods are used to evaluate these drugs will be a feasible way to obtain more knowledge, before more expensive large scale phase III studies in the clinic are initiated.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, Asbjorn Mohr Drewes, MD, *Series Editor*

Functional oesophago-gastric junction imaging

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Abstract

Despite its role in disease there is still no definitive method to assess oesophago-gastric junction competence (OGJ). Traditionally the OGJ has been assessed using manometry with lower oesophageal sphincter pressure as the indicator. More recently this has been shown not to be a very reliable marker of sphincter function and competence against reflux. Disorders such as gastro-oesophageal reflux disease and to a lesser extend achalasia still effects a significant number of patients. This review looks at using a new technique known as impedance planimetry to profile the geometry and pressure in the OGJ during distension of a bag. The data gathered can be reconstructed into a dynamic representation of OGJ action. This has been shown to provide a useful representation of the OGJ and to show changes to the competence of the OGJ in terms of compliance and distensibility as a result of endoluminal therapy.

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Key words: Oesophagogastric junction; Lower oesophageal sphincter; Competence; Distensibility; Functional imaging

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INTRODUCTION

The oesophago-gastric junction (OGJ) is located between the oesophagus and the stomach. Its dynamic action is dependant upon the intrinsic and extrinsic effects of its own anatomical structure and its position with respect to the surrounding organs in this region of the body. Despite its relationship to diseased states of the oesophagus, no objective measurement technique has been developed to quantify the normal or abnormal functioning of the OGJ. It is generally accepted that sphincters divide the gut into functional segments. They are characterized by a resting tension, generally known as muscle tone which is greater than in the two adjacent segments. It is common nowadays to give the term sphincter a functional meaning rather than its original anatomical meaning[1]. The sphincter at the lower end of the oesophagus, the lower oesophageal sphincter (LOS), forms part of the OGJ structure. At the OGJ the oesophagus opens into the stomach at the cardia. It is important that backflow of stomach secretions into the oesophagus is controlled at the OGJ. The OGJ guards against gastric acid moving up into the oesophagus, opening only transiently to allow passage of the swallowed food into the stomach. Swallowing is a demanding reflex activity[2]. The diaphragmatic hiatus, through which the oesophagus passes just at the OGJ, has a role in this valvular mechanism[3].

Several other structures are important in maintaining a barrier in the OGJ against the back flow of food and the stomach acids into the oesophagus, known as the anti-reflux barrier. The intrinsic muscles of the distal oesophagus and the sling fibres of the proximal stomach make up the internal mechanism of the LOS[4]. The muscles of the diaphragm that connect to the OGJ make up the crural diaphragm and this constitutes the external LOS mechanism. The tissue that connects the distal oesophagus to the crural diaphragm is known as the phreno-oesophageal ligament[5].

The OGJ can be distinguished from the body of the oesophagus by its behaviour pattern characteristics. There is an increase in the tone of the circular muscle in this region. The sphincter relaxes in response to a swallow and this usually precedes the arrival of a contraction wave travelling down the oesophagus. This phase of relaxation is followed by a short-lasting elevation of pressure above resting values[6]. In recent years it has been identified that the sphincter sometimes relaxes even when a swallow does not occur. These relaxations are know as transient lower oesophageal sphincter relaxations (tLOSRs)[7]. Other factors contribute to the role of the OGJ in controlling the flow of solids, liquids and gases between the oesophagus and the stomach. Pressures in the abdominal and thoracic cavities are involved as well as the exact intra-abdominal

location of the junction. Time and posture are also important factors.

THE ROLE OF THE OGJ IN DISEASE

Disorders of the OGJ in general relate to two diseased states. Conditions where the disease may be related to an OGJ not closing normally are generally referred to as gastro-oesophageal reflux disease (GORD) and conditions where the OGJ fails to relax or open, which is referred to as achalasia.

Gastro-oesophageal reflux disease

GORD symptoms are very common in the general population affecting up to 44% of the US population at least once a month and 20% of the population once a week[8,9]. It has been shown that patients suffering from GORD have a lower health related quality of life than patients with angina and mild heart failure[10]. There is solid evidence that incompetence or dysfunction of the OGJ is a primary determinant of GORD. Three mechanisms at the OGJ lead to an increased number of acid reflux events associated with GORD: tLOSRs, free reflux during periods of low LOS pressure or deglutitive relaxation of the LOS[7,11-13]. Much more needs to be learned about the dynamic action of the OGJ if we are to fully understand its role in GORD. Current treatment of GORD is with acid suppressing drugs such as proton pump inhibitors but for those refractory to pharmacological treatment surgery is often recommended. The surgery of choice for GORD is the laparoscopic Nissen fundoplication[14].

Achalasia

Achalasia is an oesophageal disorder of unknown cause characterized by aperistalsis of the oesophageal body and impaired relaxation of the lower oesophageal sphincter[2,15]. It is a relatively rare disease and in Western countries the annual incidence is in the range 0.5 to 8 cases per 100 000 people[16]. It can be difficult to make a diagnosis in some patients because of vague symptoms. Symptoms can emerge slowly and patients adapt. The diagnosis can be confirmed by endoscopy and manometry[17]. Developing a device that can profile the OGJ will contribute greatly to the understanding of this disorder, which often manifests as a permanently constricted or toned sphincter. Surgical performance of an oesophageal myotomy is the treatment of choice for some severe cases of achalasia[18]. Currently the laparoscopic oesophageal Heller-Nissen procedure is commonly used[19].

BIOMECHANICS OF THE OGJ

Biomechanics are the laws of mechanics applied to biology[20]. There is an increasing recognition of the importance of tissue biomechanical properties for function, e.g. organ geometry, resting conditions and elasticity is determinants of flow in the organ and sensory responses in the neural circuits. When dealing with biological tissues, solid mechanical parameters such as the relation between forces (membrane stress resultant, tension) and deformation (often expressed as a strain) are important to evaluate. The mechanical properties of the OGJ are important determinants of its distensibility and competence. In vitro studies have shown potential in measuring biomechanical parameters in the OGJ[21-25]. Very few methods exist for assessment of mechanical properties in the GI tract *in vivo*. One method is impedance planimetry from where the cross-sectional area (CSA) and pressure data can be gathered and the wall tension and strain in the organ can be estimated[26]. Other methods rely on the combination of bag distension and manometry with high resolution imaging techniques such as 3D-ultrasound of the gastric antrum[27] or MRI of the rectum[28]. Manometry is of course a simple biomechanical measurement. Its use will be mentioned again later.

METHODS OF EVALUATING OGJ FUNCTION

In 1964 Mann *et al*[6] suggested that the zone separating the lower end of the oesophagus from the stomach can be recognized in two different ways, by its distinguishing anatomic features and its unique patterns of behaviour. A huge amount of knowledge about the anatomy of the OGJ has been assembled over the last 40 years, but much of our limited understanding of the behaviour has been related to manometric studies using single or multiple pressure recordings or a sleeve placed in the OGJ. While manometry provides useful information on motility within the lumens of the digestive tract, it may be more restrictive in what it can tell us about sphincteric regions such as the OGJ and their dynamics. Harris and Pope found that sphincters do not necessarily need to squeeze or contract tightly to be competent and therefore resistance to distension by measurement of radial force should be the prime determinant of sphincteric strength[29]. Measurement of luminal pressure, however, is not a direct measure of muscle tone and tension. It is merely a proxy of changes in force generated by the surrounding tissues[27,30].

No clinically acceptable quantitative method exists to directly measure OGJ mechanical function. Intraluminal pressure recordings is the only objective measurement technique for determining the competence of the LOS, but it is usually the more indirect method of measuring acid reflux in the oesophagus which is the key determinant of failure of the OGJ to inhibit flow. Thus, pH-metry rather than manometry is the recommended test for GORD[31].

Usually in medicine dynamic diagnostic imaging methods can be used to visualize and evaluate anatomical structures similar to the OGJ. Currently none of these methods give sufficient dynamic detail to contribute to the routine diagnosis and treatment of diseases of the OGJ. Recent developments in material sciences and technology have allowed the OGJ's resistance to distension to be studied using parameters such as distensibility, opening patterns and dimensions and flow rate[32-34]. It is becoming apparent that these measurements may be useful in determining the success of therapeutic interventions such as surgery[35]. However what is needed is a technique with high spatial and temporal resolution for determination of sphincter geometry and mechanical factors.

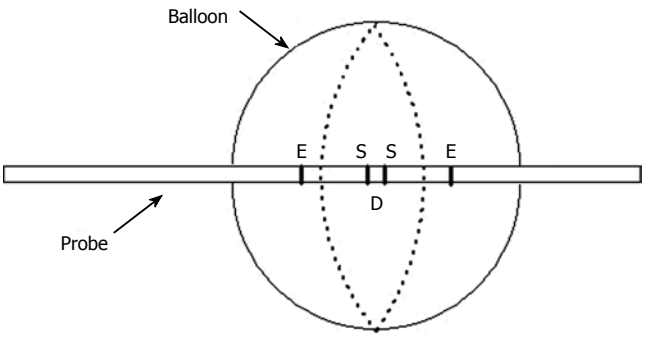

Figure 1 Sketch of a conventional impedance planimetry probe with a single set of excitation (E) and sensing (S) electrodes for the measurement of cross-sectional area in the middle of the bag. D is the distance between the sensing electrodes.

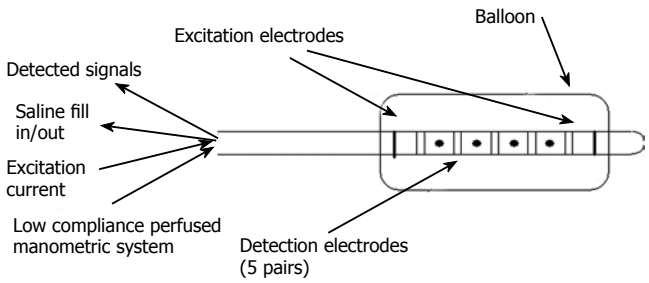

Figure 2 Diagram showing multi-electrode probe by Andersen et al for measuring 5 CSAs in the porcine rectum.

Impedance planimetry

Principle of Impedance Planimetry: Impedance planimetry (IP) is a technique established by Gregersen and coworkers for performing bag distensions in the GI tract (Figure 1). It has been used extensively to provide a single measure of CSA together with bag pressure in animal and human studies using electrical measurements inside the liquid filled bag[36]. Hence the term impedance planimetry. If we fill a polyurethane bag with saline and pass a constant current through it, the CSA is inversely proportional to the voltage across the sensing electrodes. If all other parameters are kept constant then the impedance becomes related to the CSA of the bag between the sensing electrodes.

$$V \propto \frac{1}{CSA},$$

Many studies have shown the usefulness and reliability of IP in the oesophagus of healthy volunteers and patients[37-54]. Rather than analyzing distension data based on pressure-volume relationships as is the case with the "Barostat", IP enables data on the CSA of the bag during distension to be gathered. CSA shows a better relationship to the diametrical changes in the alimentary lumen than volume[27]. However, by far most studies have been done using a single CSA recording inside the bag rather than obtaining a series of CSAs. Due to previous limitations, it has only been used in a few studies in sphincter regions[55,56]. These limitations related to the difficulties placing the measurement electrodes at the point of most interest and the fact that distending a bag in a high-pressure zone tends to displace the bag during the measurements.

Multiple measurements: A recent pilot study in the porcine rectum demonstrates how an IP probe placed in the rectum with 5 sensing electrode pairs with 2mm between pairs and 2cms between sets can detect contractile events at different levels along the longitudinal axis of the gut (Figure 2)[39,57]. This multi-electrode technique demonstrated how a balloon with a conducting liquid and a constant current source set up across two excitation electrodes can measure multiple CSAs by measuring the voltage across multiple electrode pairs within the electric field.

There are a number of potential sources of error in multiple measurement IP systems. Electrodes placed too close together will interfere with each other. The number of wires required to make the electrical connections goes up and therefore the potential for interference, liquid leaking into the probe and bad electrical connections is much.

Manometry

Manometry has been used for more than 50 years to monitor motility in the gastrointestinal tract. Since 1977 reliable methods have been available to obtain accurate measurements of force of closure of the lumens of the intestine when contracting. These system often use reliable perfused hydraulic-capillary systems where pressure at a sensor outside the body can be used to accurately measure pressure at a point along a catheter via a low flow rate perfused channel[58]. This system has been used extensively to measure force via a pressure measurement in lumens and in liquid filled balloons. Errors in manometric readings can be caused by air bubbles in the capillary lines, occlusion of the measurement site and the compliance of the recording system but in general if well set up they produce accurate readings. In more recent years catheters with solid state transducers are becoming more popular for pressure reading and these overcome some of the measurement problems.

Using manometry to determine the competence of sphincteric or valvular regions in the lumens of the digestive tract is however dubious. Dent recognized the problem of positioning the measurement point 30 years ago and proposed a solution[59] and others tried to follow suit[60]. But pressure is a measurement of force over area and this may not be a proper measure of the function of a valvular region. As suggested by Pope and Harris we cannot assume that just because a sphincter is closed that it has a contract force or in the terminology of manometry that if it has a high lower oesophageal pressure it is deemed to be competent.

Calculation of wall tension

Law of laplace: For a membraneous cylinder with transmural pressure P and radius r the circumferential tension T can be given by :

$$Pr = T$$

Use of Laplace's law is based on the assumption that the geometry of the segment is circular and cylindrical and that the wall thickness is very small.

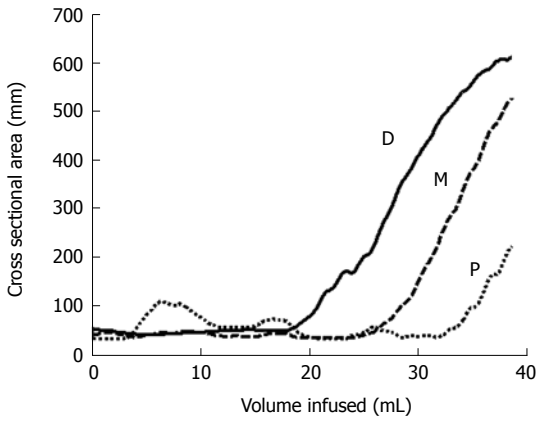

Figure 3 Plot showing the changes in the CSAs at the proximal (P), middle (M) and distal (D) electrode pairs as the balloon is distended at a constant flow rate indicated by the increase in volume.

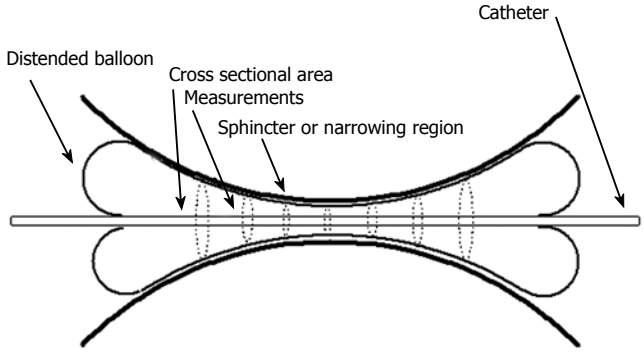

Figure 4 An example of how a probe which can measure 8 CSAs could profile the geometry of the narrowing region of a bodily sphincter.

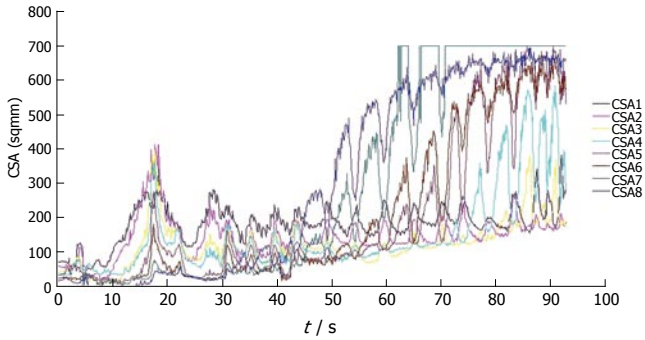

Figure 5 Plot showing changes in the 8 measured CSAs during bag distension at 40 mL/min. CSA1 is the most distal and CSA8 is the most proximal.

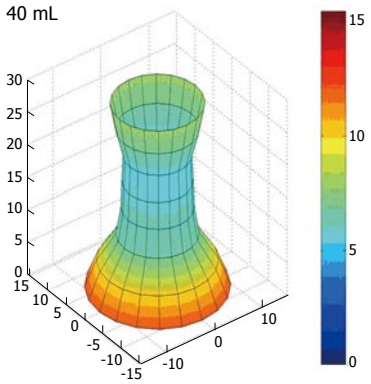

Figure 6 Three dimensional reconstruction of the OGJ geometry in a human volunteer after distension in to a volume of 40 mL. All dimensions are in millimeters. Colour change is indicating increased radius.

Measurements using multi-electrode Impedance planimetry in the oesophagogastric junction

We have demonstrated that a multi-electrode probe with a cylindrical bag mounted and filled with saline can be placed in the OGJ and that data on a number of CSAs in this area can be measured[49]. Figure 3 shows results of 3 CSA measurements 8 mm apart measured from distension of a bag in the OGJ of a healthy volunteer. The plot shows how the most distal CSA (D) measurement increases more rapidly than the middle CSA (M) as a constant volume of saline is filled in to the bag. For the most proximal CSA (P) the converse is true. This suggests that distally the OGJ is more compliant than proximally. This work also demonstrated for the first time how wall tension in the OGJ can be estimated from simple distension measurements. This preliminary version of the multi-electrode technique demonstrated that a probe can be placed in the OGJ and that data can be successfully collected. However, there were limitations such as the fact that there were only three measurements made. A narrowing region such a sphincter can not be profiled very well with three CSA measurements unless the exact position of the sphincter is located and maintained. Another limitation is the outer diameter of the catheter on which the bag is mounted. In this case it was 4.5 mm. If the sphincter is to be distended from as close to the closed position as possible then a smaller catheter needs to be used.

Figure 4 shows how a probe capable of measuring 8 CSAs could be used to profile a sphincter region. It is quite obvious that a larger number of sensing pairs could be more useful in providing a geometric profile. Consequently a more elaborate probe for measurement of up to 8 CSAs in the region of the oesophago-gastric junction was developed. This probe can measure CSAs at 4 mm intervals through the OGJ thus spanning a measurement range of 28 mm.

Figure 5 shows the data from the 8 CSAs during a bag distension at a fixed flow rate of 40 mL/min to a volume of 60 mL. The information in this format can be difficult to decipher. A probe to make these measurements has been validated and test in a healthy control subject[57]. Since the OGJ is such a dynamic organ it is its functional activity which can be more of interest. Hence it is possible to generate an illustration of the geometric changes in the OGJ.

If we introduce the idea of demonstrating the CSA changes dynamically as reconstructed data we introduce a new concept known as the functional lumen imaging probe (FLIP). The concept suggests that by gathering data on the dynamic changes in the geometry of the OGJ during normal physiological changes we can represent these changes by recreating the geometry in a three-dimensional image. The FLIP technique can be used to monitor geometric changes in the behaviour of the OGJ during bag distension as can be seen in Figure 6. Here the dynamic data is reconstructed demonstrating the effect of the balloon distension on the amount of OGJ opening.

FLIP can quantify changes in compliance of the OGJ after endoscopic suturing at the OGJ in a pig model[61]. Figure 7 indicates how the geometric reconstruction can be used to indicate changes in the profile of the OGJ after endoscopic plication. The pressure is higher, the CSA is smaller and subsequently the wall tension has increased after the plications are inserted. These measurements indicate that the OGJ becomes less compliant after plication. No direct measure of the effects of these therapies on the OGJ has been demonstrated before. Recent studies have expressed concerns about the efficacy and durability of these procedures[62-65]. FLIP may in fact reveal new information on the mechanism of action behind these new reflux therapies. In effect it suggests that endoscopic suturing augments the compliance of the OGJ to make it less compliant. Perhaps patients could now be selected better for these procedures if their compliance can be confirmed to be abnormal in the first place and maybe the success of the procedure could be determined directly at the time of the procedure so for example more stitches can be added if necessary.

Since GORD patients are such a large group perhaps this is the most important area for FLIP to be developed. The probe can distinguish between patients for reflux surgery and normal controls. These are of course the extreme group of patients. Further studies are of course necessary on larger cohorts of patients and controls.

We have recorded with the FLIP the compliance of the OGJ intra-operatively during oesophageal myotomy for achalasia (as yet unpublished findings by the authors). FLIP offers a more sensitive and possibly a better tool than manometry and may be used for evaluating effects of surgery during the procedure as opposed to using manometry more as a blunt instrument before and after as used by Arain *et al*[66]. FLIP could provide a valuable new tool for the oesophageal surgeon.

Summary

Work already carried out shows for the first time that CSA and pressure measurements in the OGJ can be used to measure distensibility by an entirely new technique. It demonstrates how the already established technique of impedance planimetry can be used to measure geometry in vivo in the human oesophagus. It also shows that there is validity of these new measurements, their appropriateness for use in sphincteric regions in the body and their comparable accuracy has been shown with ultrasound. It has been indicated how the measurement could be used practically. Safety and efficacy has been demonstrated. The work also demonstrates how this new measure could be used as a functional imaging technique. Preliminary studies have shown potential applications of the probe. It could prove to be a very valuable tool in endoscopic therapies. It can distinguish between normal and a stitched OGJs in live pigs.

As a resistance to distension test this better describes the sphincteric action of the OGJ than tonic contraction. Even advanced manometry uses such as volume vector analysis, the Dent Sleeve or the Sphinctometer all rely on the principal that there is a force of contraction always present when the sphincter is closed[59,60,67-70]. With FLIP this assumption is not made and it presents more of a challenge test to the sphincter. The technique also represents a more direct technique to evaluate the OGJ using opening patterns with manometry and intraluminal impedance[71].

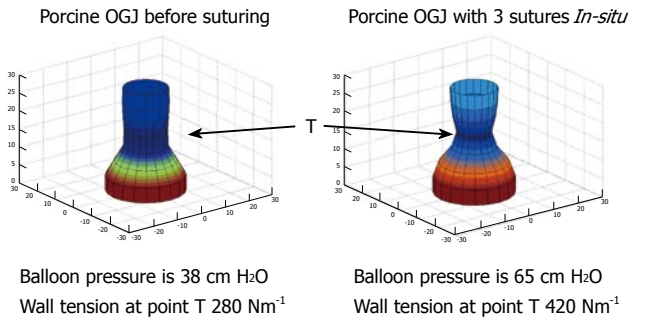

Figure 7 Geometric profile of the porcine OGJ before and after the placement of 3 sutures in the OGJ. T indicates the point where wall tension was measured.

Some other techniques for measurement of resistance to distension by other investigators are promising and confirm that the principals describe here have great potential for merit. However these other approaches are limited in a number of ways. The technique described by Pandolfino, although it is similar in its measurement design to FLIP, it requires fluoroscopic imaging[32,33,35]. It also depends on the pressure being controlled. Pressure control is difficult to achieve by increasing and reducing volume through a narrow catheter. Using pressure-volume data from the barostat to quantify distensibility does not a good basis in the application of the physical sciences. The bag on a barostat can expand in all directions we cannot be sure that an increase in the volume is reflected by a change in the geometry of the OGJ. This suggest that using this technique is of limited use[34]. Our findings in patients with achalasia confirmed this.

Perspectives

FLIP represents the first validated data on a probe capable of measuring OGJ compliance in a simple and time-saving procedure. It represents the opportunity to define the exact role of the OGJ in dysmotility and disease. As a new technique rather than representing a completed work it opens up a new door and perspectives on the junction whose function has been the subject of debate for many years. Future work and improvements in electronics and material sciences will mean that the resolution of the FLIP can be increased dramatically. Design materials have been carefully chosen to minimize signal error. The lower oesophageal sphincter or the oesophagogastric junction are often cited as being the prime causes of reflux disease, yet despite this very few research groups are investigating new ways to evaluate this dynamic structure in the gastrointestinal tract[32,34].

Much work has been done in extending manometric measurements enhancing pH measurement techniques to further evaluate the effects of acid in the oesophagus[72] and developing new techniques for measuring bolus transit

both down to the stomach and back up into the oesophagus using intra-luminal impedance[71]. However, none of these techniques have overcome the fundamental question of how the OGJ function can be quantified.

FLIP may provide the answer to this question. In a way trialing the probe in the OGJ was probably the most challenging and perhaps experiments in another sphincteric region would have been more straight-forward where the dynamic activity would be slower. FLIP may have a role in determining biomechanically the root cause of diseases. It can be used for evaluating therapy for example endoscopic and surgical therapy for reflux. It may be possible to use it intra-operatively during Nissen fundoplication, for example where the tension of the wrap is extremely important. It may also be possible to explore other sphincters in the body with FLIP so that the relationship between their biomechanical properties and their relationship to disease can be further defined.

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TOPIC HIGHLIGHT

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Gastric accommodation assessed by ultrasonography

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Abstract

Gastric accommodation is important for the understanding of the pathophysiology in functional dyspepsia and is also relevant for symptom generation in other disorders. The term gastric accommodation has at least three different meanings: The accommodation *process*, the accommodation *reflex*, and the accommodation *response*. The gastric accommodation *process* is a complex phenomenon that describes how the size of the gastric compartment changes in response to a meal. The electronic barostat is considered the gold standard in assessing gastric accommodation. Imaging methods, including MRI, SPECT, and ultrasonography may also be used, particularly in patients who are stress-responsive, e.g. functional dyspepsia patients, as a non-invasive and less stress-inducing method is favourable. Ultrasonography satisfies these criteria as it does not by itself distort the physiological response in stress-responsive individuals.

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Key words: Ultrasonography; Gastric accommodation; Stomach; Barostat; Gastric relaxation; 3D ultrasound

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INTRODUCTION

Gastric accommodation has become a key mechanism for the understanding of the pathophysiology in functional dyspepsia[1,2] and may also be relevant for symptom generation in other disorders[3-6]. Several methods can be used to assess gastric accommodation[7]. The electronic barostat is considered to be the gold standard method for measuring proximal gastric accommodation[8,9]. By applying the barostat, gastric tone is estimated by recording variations in the volume of air within an intragastric bag that is maintained at a constant pressure by the instrument. Accommodation of the proximal stomach is supposed to be reflected in changes of gastric tone. However, the method is invasive, bothersome and affects normal gastric motility[10]. The bag may also alter intragastric distribution and emptying of the meal the direct stimulus imposed by the bag itself on the stomach wall results in increased antral distension[11]. Therefore, non-invasive methods as MRI, SPECT, and ultrasonography are warranted for the study of gastric accommodation.

MRI is a safe and accurate technique to image the entire stomach with detail[12]. After ingestion of a test meal labelled with gadolinium as an MRI marker, images of the gastric region are acquired, typically using a Spin Echo T1-weighted sequence. The MRI technique permits three-dimensional reconstruction of the stomach, enabling total gastric volume measurements during fasting and postprandially. The ratio of these measurements is used to quantify the accommodation response. Single Photon Emission Computed Tomography (SPECT) uses ^{99m}Tc uptake into the gastric mucosa for visualisation and volume estimation of the gastric compartment[13]. Comparison of SPECT data with barostat measurements showed that SPECT is able to detect changes in intragastric volume but unable to detect changes in gastric tone and hence accommodation[14].

GASTRIC ACCOMMODATION

Conceptually, the process of gastric accommodation has been explored for over 100 years, evaluating relaxation of the proximal stomach[15]. Receptive relaxation has been defined as a vagal reflex, which dilates the corpus-fundus region of the stomach in response to swallowing, oesophageal stimulation, or vomitation[16-18]. Adaptive relaxation is a intrinsic vagovagal reflex that enables the stomach to increase its volume markedly without a corresponding increase in pressure[19,20]. Adaptive relaxation enables the stomach to maintain a low balanced pressure and to continuously adapt its volume to its content.

However, the term gastric accommodation is too

complex to embrace in one definition, mainly because it is utilized in at least three different meanings in the literature: The accommodation *process*, the accommodation *reflex*, and the accommodation *response*. In addition, the term is often used in an everyday language expressing that "the stomach accommodates a meal". Similarly, it was also used by Paton and Vane in 1963 to describe gastric "expansion to accommodate the fluid"[21]. The gastric accommodation *process* is a complex phenomenon that describes how the size of the gastric compartment changes in response to a meal[22]. It involves both intra- and extragastric reflexes. The extragastric reflexes comprises both receptive relaxation and intestino-gastric reflexes elicited by balloon distension or nutrient infusion in the duodenum[8,23]. The intragastric reflexes embrace at least adaptive relaxation and relaxation of the proximal stomach induced from antral reflexes[24-26].

Kelly[27] described the accommodation *reflex* when he demonstrated how intragastric pressure was regulated by gastric "accommodation to distension". The accommodation reflex is elicited when continuous volume expansion of the proximal stomach reaches a certain level, thus inducing relaxation of smooth muscles of the gastric wall in order to prevent further increase in pressure. This physiological phenomenon is a reflex arc consisting of a mechanoreceptor in the gastrointestinal wall, a neuro-muscular pathway through ENS with a reflex centre, and a well-defined response in the effective organ. The reflex is probably vagally mediated but local axon reflexes may also be involved[20]. The term accommodation *response* is the effect of the *reflex* or the result of the *process* and this is a precise notion for what we intend to measure with different methods.

IMAGING METHODS

A crucial question is whether imaging methods, as MRI, SPECT or ultrasonography, actually can be compared to the measurements made by the barostat. In our opinion, imaging methods and the invasive barostat method do not measure the same aspects of the gastric accommodation *process*. Thanks to its close contact with the gastric wall the barostat bag adjusts to changes in proximal gastric pressure by changing the intrabag volume. Thus, changes in volume are believed to reflect changes in muscle tone of the wall. However, the quantitative change in volume seen during barostat examinations is only valid during barostat studies using exactly the same equipment and positioning technique. Imaging methods, however, visualize directly the size of the gastric compartments, thus giving an indirect measure of relaxation and contraction. The volume change seen using imaging can thus be explained by additional secretion, air retention or most probable changes in gastric emptying.

The gastric meal accommodation *process* has two components: Passive meal distension of the gastric compartments and active muscle relaxation of the gastric wall. The first component is best measured with imaging methods whereas the barostat is best suited for studying the second component. Imaging methods at this stage, do not distinguish between enlargements of the stomach due to reflex relaxation or due to meal-induced distension; it just measures the totally accommodated volume. Accordingly, it may not be adequate to compare imaging methods to the barostat for validation of gastric accommodation. Gastric accommodation depends on neuromuscular factors and hence it is also a matter of evaluation of the mechanical properties of the stomach. In this sense the barostat merely detects the existence of change in wall tone, but cannot as the imaging methods, provide data on the distribution of the volume and the normal behaviour of the gastric wall. In biomechanics, it is essential to understand the geometry of the organ and the forces and deformation in different directions in order to better understand the active-passive muscle function and mechano-sensation in health and in diseases such as the functional disorders. This warrants 2D and 3D image analysis of the gastric compartments.

The different imaging methods exhibit different spatial and temporal image resolution, and these factors strongly influence accuracy in volume calculation. MRI and in particular SPECT imaging have poor spatial and temporal resolution compared to ultrasonography. Ultrasonography can provide temporal resolution above 100 fps, if necessary, and a spatial resolution on sub-millimetre level. Also, the "stress-factor" of the imaging methods should be considered in this context because dyspeptic patients in general and vagal reflexes in particular are very sensitive to psychological stress. Everyone who has been inside a narrow, noisy MR scanner knows how frightening this can be. Naturally, the stress-factor is also substantially involved in studies using the barostat. Simply because functional disorders are so strongly associated to psychological factors, the examination should be performed in a quiet and relaxing atmosphere with a minimum of distress. Ultrasonography satisfies these criteria as it is non-invasive and does not by itself distort the physiological response in stress-responsive individuals. Moreover, due to gravity playing a central role in the propulsion of gastric content, the study of meal accommodation should preferably be performed in a "natural position" such as sitting in a chair. Therefore, methods that enable patients to be seated have an advantage over methods requiring patients to be in supine position during the examination.

Ultrasonography

Ultrasonography, a clinical method that is widely available, has shown applicability and validity for the study of gastric accommodation[28]. The 2D method developed by Gilja *et al*[29] has been used to study gastric accommodation in functional dyspepsia[1,30], diabetes[4], in patients with reflux oesophagitis[5], in liver cirrhosis[31], and in children with recurrent abdominal pain[6]. This 2D method has also been applied to study pharmacological intervention[32] and the effect of a barostat bag in the stomach[11]. Another advantage with 2D ultrasonography is its clinical applicability; it can easily be performed bedside and repeated numerous times in the same subject. At Haukeland University Hospital we have for 20 years used The Ultrasound Meal Accommodation Test (U-MAT) for the work-up of patients with dyspepsia (Table 1). Our mainstream clinical protocol consists of a standard soup meal (500 mL), ultrasound

Table 1 The ultrasound meal accommodation test

Time	Protocol
Fasting	Ordinary ultrasound examination of the liver, gallbladder, biliary tract, spleen, pancreas, kidneys, and large vessels.
Fasting	Evaluation of symptoms by VAS.
Fasting	Assessment of motility pattern (phase 1-3) by observing the pattern of contractility in the antrum.
Fasting	Measurement of area of the distal stomach (AA).
Fasting	Visualisation of the proximal stomach to explore whether it has content.
Meal ingestion	500 mL of preheated meat soup is ingested in 4 min at a constant speed.
2 min pp.	Measurement of the sagittal area (SA), the oblique frontal diameter (OFD), and the antral area (AA).
5 min pp.	Postprandial symptom evaluation.
10 min pp.	SA, OFD, and AA measurement.
20 min pp.	SA, OFD, and AA measurement.
30 min pp.	SA, OFD, and AA measurement.

The ultrasound meal accommodation test (UMAT) was developed at Haukeland University Hospital on the basis of close interaction between scientific and clinical work in patients with dyspepsia. Before entering the protocol, the patients have been carefully worked-up with history, physical examination, blood tests, testing for H pylori, and upper endoscopy. In some cases, additional examinations are performed to rule out organic causes for their symptoms. The protocol presented here is the mainstream clinical protocol. In scientific studies other elements are often added. A 500 mL liquid meal of commercial meat soup (Toro × clear meat soup, Rieber & Søn A/S, Bergen, Norway) containing 1.8 g protein, 0.9 g bovine fat, and 1.1 g carbohydrate (83.68 kJ) is ingested over a period of 4 min. The soup is preheated and then cooled to 37 ℃ to improve imaging quality by reducing the amount of air bubbles. Psychometric evaluation is also performed.

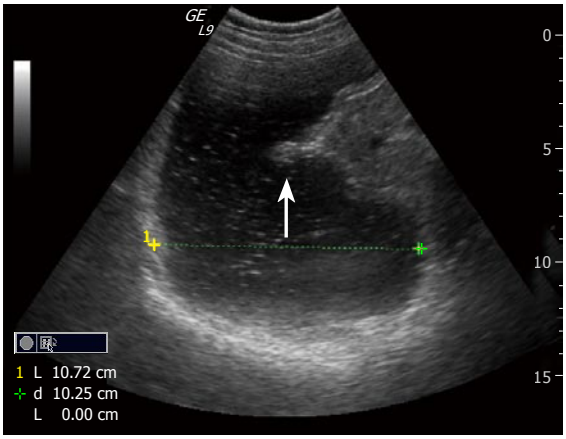

Figure 1 Ultrasonogram showing an oblique frontal section of the proximal stomach in a patient with dyspepsia referred for Ultrasound Meal Accommodation Test (UMAT). The transversal diameter is measured indicating the size of the postprandial proximal stomach and hence indirectly degree of accommodation. Interestingly, one can also observe a gastric contraction (white arrow) in the posterior angular part and progressing towards the antrum.

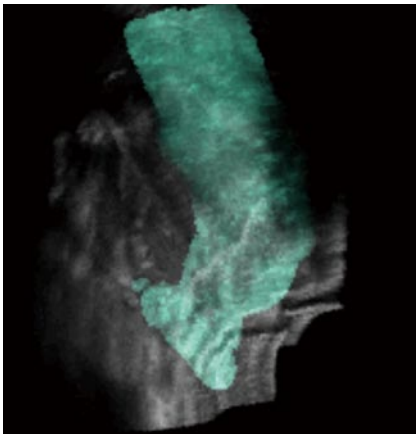

Figure 2 3D image reconstruction based on ultrasound acquisition with a magneto-based position- and orientation measurement system (POM) using GE Logiq 9. The 3D image is overlayed the ordinary gray scale values of ultrasonograms.

scanning of the proximal and distal stomach using predefined scan sections (Figure 1), calculation of size and volumes of the gastric compartments, evaluation of symptoms and psychological assessment. In our experience, ultrasonography used in this context adds valuable clinical information to the management of these patients.

There are few methods that are capable of measuring all parameters of gastric motility simultaneously. However, many authors have demonstrated that ultrasonography can be used to study not only gastric accommodation, but also gastric emptying[33-39], gastric configuration[40-42], intragastric distribution[43], antral motility[44-46] and distensibility[47,48], transpyloric flow[44,49-51] and strain of the gastric wall[42,52,53]. Of the present available methods, we would argue that ultrasonography is the method that has been shown to estimate most parameters of gastric motility, many of these also simultaneously. Furthermore, we claim that ultrasonography has the strongest potential as a clinical tool compared to MRI, SPECT, and scintigraphy, and particularly in patients with functional disorders.

3D ultrasound

For better visualization of the total stomach and improved calculation of gastric volumes, 3D ultrasound imaging of the gastric compartments was developed[43,54,55] It is important that validation of an imaging method to study gastric accommodation also include evaluation of its accuracy and precision in volume estimation. In our lab, we have utilized two different systems for acquisition of 3D ultrasound images and both systems have been validated *in vitro* and *in vivo*. First, we developed a mechanical system with the advantage of an accurate motorized movement of the ultrasound transducer in a tilting fashion. This 3D system demonstrated excellent accuracy and precision *in vitro*[56,57] and good agreement with MRI *in vivo*[58]. Subsequently, we applied a magneto-based system (Bird sensor, Ascension Technology Corp., Burlington, VT, USA) that enabled greater flexibility during scanning and larger volumes like the total gastric compartment to be captured (Figure 2). The Bird system also showed excellent accuracy *in vitro*[43,59-61] and in volume measurement of a thin-walled fluid filled bag in the human stomach the agreement with true volumes was excellent[62]. Accordingly, 3D ultrasonography delivers high resolution, is accurate in volume estimation and is a well-suited tool to monitor volume changes of the stomach during the *process* of accommodation. However, we acknowledge its limitations: impairment of image quality with air in the GI tract, mainly for use with liquid meals, and scanning of very obese subjects (fortunately dyspeptics are not often obese).

Abnormalities of gastric accommodation are a significant finding in several patients groups, not only in patients with functional disorders. Comprehension of gastric geometry, the forces and deformation during meal accommodation, and the active-passive muscle function is important in functional diseases[63]. Of the present available methods, we would argue that ultrasonography is the method that has been shown to estimate most parameters of gastric motility, many of these also simultaneously[64]. Furthermore, we suggest that ultrasonography has the strongest potential as a clinical tool, particularly in patients with functional disorders.

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TOPIC HIGHLIGHT

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Functional findings in irritable bowel syndrome

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Abstract

The pathophysiology of IBS is complex and still incompletely known. Both central and peripheral factors, including psychosocial factors, abnormal GI motility and secretion, and visceral hypersensitivity, are thought to contribute to the symptoms of IBS. Several studies have demonstrated altered GI motor function in IBS patients and the pattern differs between IBS subgroups based on the predominant bowel pattern. Few studies have so far addressed GI secretion in IBS, but there are some evidence supporting altered secretion in the small intestine of IBS patients. Visceral hypersensitivity is currently considered to be perhaps the most important pathophysiological factor in IBS. Importantly, several external and internal factors can modulate visceral sensitivity, as well as GI motility, and enhanced responsiveness within the GI tract to for instance stress and nutrients has been demonstrated in IBS patients. Today IBS is viewed upon as a disorder of dysregulation of the so-called brain-gut axis, involving abnormal function in the enteric, autonomic and/or central nervous systems, with peripheral alterations probably dominating in some patients and disturbed central processing of signals from the periphery in others.

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Key words: IBS; Gastrointestinal motility; Visceral hypersensitivity; Gastrointestinal secretion; Intestinal gas; Brain-gut axis; Brain imaging; Autonomic nervous system

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INTRODUCTION

Irritable bowel syndrome (IBS) is characterized by abdominal pain and/or discomfort related to abnormal bowel habits[1]. It is probably the most common disorder encountered by gastroenterologists[2] and also the most common gastrointestinal disorder seen in primary care[3]. A problem in the management of IBS is that there are no structural or biochemical disease markers for these patients. Therefore, various diagnostic criteria have been used to define IBS in clinical practice and, more importantly, in research settings[1]. In the Western world, IBS appears to affect up to 20% of the population at any given time[4-6], although the prevalence figures vary substantially depending on the definition of IBS[7,8]. Due to its high prevalence and, for many patients, chronic nature and incapacitating symptoms the cost of IBS to society is substantial[9].

Despite being very common, the pathophysiology of IBS is far from understood. IBS has for a long time been considered a disorder of disturbed gastrointestinal motility, although uniform motility patterns in IBS have been hard to define. Moreover, motility findings have been difficult to relate to symptoms, especially abdominal pain[10,11]. Beginning in the early 1970s, but progressing rapidly in the 1990s were theories that visceral hyperalgesia independent of enhanced motility could explain the painful symptoms of IBS[12]. More recently, a more integrated model of brain-gut interactions proposes that the central and enteric nervous systems interact and modulate enhanced motility, abnormal sensation and autonomic reactivity: the brain-gut axis. In context with this model, psychosocial factors, rather than being a cause of IBS, are regarded as contributing to the predisposition, precipitation and perpetuation of IBS symptoms, and affect the clinical outcome[13]. However, despite the increasing knowledge of the pathophysiological and clinical aspects of IBS, there are a substantial number of unresolved issues.

During recent years the interest in finding relevant alterations in patients with IBS in the function of the gastrointestinal tract, as well as in the central and autonomic nervous system has increased. The trend is now that there is less focus on pure gastrointestinal alterations and instead more focus on interactions between exacerbating factors and gastrointestinal function, as well as on interaction between different parts of our nervous system and the gastrointestinal tract. This article will review the literature

on findings in IBS patients supporting disturbed function in the gastrointestinal tract as well as its interaction with the central and autonomic nervous systems.

ALTERED GASTROINTESTINAL MOTILITY

Esophageal and gastric motility

Most studies of motility in functional bowel disorders have focused on the large and small bowel, although there are also reports on disturbed motility in the esophagus and the stomach. Patients with IBS demonstrate, for instance, lower pressure in the lower esophageal sphincter and more esophageal contraction abnormalities than healthy subjects[14]. Furthermore, patients with contraction abnormalities in the esophagus often report symptoms compatible with IBS[15]. There are several studies of gastric emptying in patients with IBS. These have come to somewhat different conclusions, with some of the older studies being unable to detect an abnormality[16-18], whereas some of the more recent studies found abnormal gastric emptying in at least a subset of IBS patients[19-22]. Moreover, the presence of gastroparesis in IBS was found to be related to small bowel dysmotility[23]. In a recent large-scale study, delayed gastric emptying was found in IBS patients with overlapping dyspepsia, but not in those without dyspeptic symptoms[24]. Some studies have also used electrogastrography (EGG) in patients with IBS finding a nice correlation between lack of increased postprandial EGG amplitude and delayed gastric emptying in a subset of IBS patients[25], whereas another study found EGG abnormalities only in IBS patients with concurrent dyspeptic symptoms[26]. The conclusion of these studies seems to be that altered esophageal and gastric motor function is more related to the presence of upper GI symptoms than to IBS per se, even though a significant overlap between upper GI symptoms and IBS exists[27].

Small intestinal motility

The motor patterns of the small bowel in man are rather well characterized[28], but marked inter- and intraindividual variations make interpretation difficult[29,30]. In the late 1970s technological advances made it possible to accurately record small bowel motility in man. Thompson and co-workers, using a radiotelemetry capsule system, reported abnormal small-bowel motility in a patient with "irritable colon"[31], but a follow-up study using a similar recording system failed to confirm this[32]. Subsequent studies have focused on the periodicity of the migrating motor complex (MMC) (i.e. the interval between successive MMC cycles), as well as finding specific motor patterns in IBS and relating these patterns to symptoms. In diarrhea prone subjects, the periodicity of the MMC during daytime has been found to be shorter[33,34], but longer in IBS patients with predominant constipation[35], although these are not consistent findings[36,37]. Increased frequency of clustered activity has been found in some[33,34,38], but not all studies[36,37,39]. Of more interest perhaps, are correlations between symptoms in IBS patients and certain motility patterns, such as clustered activity (e.g. "discrete clustered contractions") and prolonged propagated contractions[33,34], but likewise, these findings have been partly[37] or totally[36] rejected by others. Furthermore, IBS patients have been found to have enhanced perception of physiological intestinal motility, such as the activity front[40]. There also appears to be a relationship between heightened sensitivity to distension of the small intestine and postprandial jejunal motor abnormalities in IBS patients[41]. An increased contraction frequency postprandially has been demonstrated in diarrhea and constipation predominant IBS[42], and in a study by Schmidt et al diarrhea prone IBS patients had more aborally propagated contractions in phase II and postprandially than the controls[37]. The contraction amplitude has, if anything, been shown to be increased in IBS patients with diarrhea[37] and reduced in those with constipation[35,42]. Our group has used high-resolution manometry in the small intestine with closely spaced recording points to evaluate the propagation pattern of individual pressure waves[43]. A study in IBS patients and healthy controls disclosed a higher proportion of duodenal individual pressure waves that propagated in a retrograde direction in IBS, especially in the postprandial phase. Moreover, when combining this high-resolution analysis with more conventional manometry analysis, abnormal findings were found in the majority of IBS patients[38].

Motility disturbances of the small bowel have also been demonstrated indirectly in transit studies, with rather uniform findings of accelerated transit through the small bowel in diarrhea predominant IBS[44,45] and delayed transit in constipation predominant IBS[18,44]. Small intestinal bacterial overgrowth (SIBO) is a condition caused by an abnormal number of bacteria in the small intestine, which is a common complication of severe small intestinal motor dysfunction[46,47]. Recently, provocative studies, using the lactulose hydrogen breath test[48], have proposed that SIBO is present in approximately 80% of IBS patients[49] and that this alteration responds favourably to antibiotic treatment with symptom improvement[50]. Moreover, the presence of SIBO in IBS patients has been proposed to be related to small intestinal motor abnormalities[51]. However, contradictory results exist and the relevance of SIBO in IBS is still open to debate[52-54].

To conclude, there is evidence of disturbed small bowel motility in IBS patients as a group, but no uniform motility pattern has been found and a consistent correlation between motility findings and symptoms has been difficult to demonstrate. Whether the observed motility abnormalities of the small intestine in IBS are due to factors related to the enteric or the central nervous system is not clear, but there is evidence to suggest that both may be true[35,39,55].

Colonic motility

Considerable effort has been made to find a specific abnormality in the myoelectrical and/or motor patterns of colon in IBS. However, a primary role for colonic dysmotility in the pathophysiology of IBS has been hard to demonstrate. Some of these difficulties may be explained by the fact that myoelectrical and motor events from the colon are less predictable and less well studied than corresponding events elsewhere in the gastrointestinal tract[56,57]. In the 1970s Snape and colleagues reported on

abnormal colonic myoelectrical activity in IBS (3 cycle/min pattern)[58,59], but subsequent studies failed to confirm this pattern to be specific for IBS[60,61]. Instead, since this pattern was demonstrated to be equally common among psychoneurotics without gastrointestinal symptoms it was suggested that this abnormality represented a colonic response to stress rather than being related to IBS[62]. Other techniques have focused on colonic spike patterns and demonstrated variations in the incidence of these[63], but whether these findings simply represent a non-specific response to diarrhea is not clear, since appropriate non-IBS controls have not been studied.

Several studies of basal colonic motility with intraluminal pressure recordings have not been able to demonstrate an abnormal pattern in IBS as compared to appropriate controls or differences between IBS patients with different symptomatology[17,64], but this has to some extent been contradicted in recent studies of colonic motility[65,66]. Some of the earlier studies were, however, able to detect frequent sigmoid contractions with high amplitude in association with pain reports from some of the IBS patients studied ("spastic colon"), although no group differences in rectosigmoid motility between IBS patients and controls could be detected[67]. Also, some recent studies have found an increased frequency of high-amplitude propagating contractions (HAPC) in the colon in non-constipated IBS patients[65,68,69], and more interestingly an association between pain episodes and HAPCs[65,70], possibly related to the presence of visceral hypersensitivity in IBS. Somewhat more consistent has been the finding that IBS patients seem to have an exaggerated and prolonged postprandial myoelectrical and motor response than healthy volunteers[17,65,71,72], even though negative studies also exist[66,73].

The normal motor response in the colon after nutrient administration is an increase in phasic motor activity and a progressive increase in tone as well[74]. At least two studies have failed to detect an abnormal colonic tone response after meal intake in IBS patients[66,75]. However, there are some recent data supporting altered gastrointestinal reflex activity in IBS patients, by demonstrating abnormal rectosigmoid tone response following a low-caloric meal[76], or as an attenuated rectal tone response to colonic distension ("the colorectal tonic reflex")[77,78]. Hopefully these interesting studies will be followed by other investigations evaluating gastrointestinal reflex activity within the gastrointestinal tract, thereby providing new important information regarding neuromuscular function within the gastrointestinal tract in IBS patients. As in the small intestine motility alterations have also been demonstrated using transit studies. Transit through the colon influences the bowel habit of the patient[79,80], and differences exist in colon transit between IBS subgroups based on the predominant bowel habit[44,81].

To conclude, there is some evidence to suggest a disordered colonic motility in IBS, even though no uniform colonic motor pattern of this patient group is evident. Of great relevance to the symptomatology is probably the exaggerated colonic motor response to physiological stimuli such as food and, perhaps also stress and emotions in IBS[61,82], which together with an enhanced visceral sensitivity might be deleterious for these patients. Alterations in gastrointestinal reflex activity in IBS patients seem plausible, but follow-up studies are needed.

ABNORMAL GAS HANDLING

In patients with IBS complaints of "too much gas" causing abdominal pain and bloating is very common, and this has been proposed to be secondary to disordered intestinal motility in combination with an abnormal GI sensitivity, rather than caused by abnormal volume or composition of intestinal gas[83]. However, an abnormal colonic fermentation in IBS has been demonstrated in one study[84], but needs to be repeated by other groups. Of great interest, patients with IBS complaining of bloating has been found to have impaired transit of exogenous gas loads, and the following gas retention reproduced their symptoms[85]. Importantly, this abnormality can be modulated by nutrients[86, 87], physical activity[88] and body posture[89]. In follow-up studies using scintigraphy the small intestine seemed to be responsible for the ineffective gas propulsion in patients complaining of bloating[90], and altered gastrointestinal reflex activity in IBS patients might also be involved in the abnormal gas handling[91].

These findings tell us that IBS patients do not necessarily need to produce more gas in order to have gas related symptoms, but these may instead be due to motor dysfunction, resulting in a transport problem, and an enhanced visceral sensitivity.

ALTERED GASTROINTESTINAL SECRETION

Few studies have so far addressed GI secretion in IBS, partly due to methodological problems in assessing gastrointestinal secretion. However, there are some evidences supporting altered secretion in the small intestine of IBS. An old study demonstrated that patients with IBS have enhanced intestinal secretion in response to perfused bile acids in the ileum, relative to controls[92]. Our group has used potential difference measurements in the small intestine as an indirect measure of secretion in the small intestine[93], and in preliminary studies found an enhanced reactivity of the secretory component of the MMC in the small intestine in diarrhea-predominant IBS and to some extent also in constipation predominant IBS[94]. Ongoing studies will further evaluate the importance of secretory abnormalities in patients with IBS and the relevance for symptoms.

VISCERAL HYPERSENSITIVITY

Since James Ritchie in 1973 demonstrated colonic hyperalgesia by inflating balloons in the sigmoid colon in patients with "the irritable colon syndrome"[95] numerous reports on visceral hypersensitivity in IBS have appeared. Lowered perception thresholds for balloon distension in IBS patients have not only been demonstrated in the rectum[96] and colon[97], but also in the esophagus[98,99], stomach[100] and the small intestine[101,102]. These findings support a generalized enhancement of gastrointestinal sensitivity in IBS patients. However, a recent investigation has implicated that the hypersensitivity seems to be pan-intestinal for

those with multiple sites of their symptoms, whereas it was restricted to a specific organ in those with more localized symptoms[103]. Whether IBS patients have a general hypersensitivity remains debatable since divergent results exist regarding somatic sensitivity[104-106]. Patients with IBS also show an altered viscerosomatic referral pattern as another indicator of disturbed processing of viscerosensory information[107]. However, visceral perception is not abnormal in all patients with IBS[100,108] and there is a considerable overlap in colonic pain thresholds between IBS patients and healthy controls [109]. Moreover, the relationship to the symptom profile of the patient is controversial[108,110-112].

In an attempt to find a biological marker for IBS, Mertz et al evaluated different aspects of rectal perception in a group of IBS patients. They found that 94 percent of patients showed altered rectal perception in the form of lowered thresholds for aversive sensations (discomfort), increased intensity of sensations or altered viscerosomatic referral[96]. Also, a more recent study has come to the same conclusion and proposed that rectal sensitivity testing is useful to confirm the diagnosis of IBS and to discriminate IBS from other causes of abdominal pain[113]. The proposal that altered rectal perception is a biological marker for IBS has, however, been questioned and the importance of psychological factors in visceral perception has been stressed instead[114]. Psychological factors might certainly be of great importance in studies dealing with visceral sensations, and response bias[115] may influence results from balloon distension studies. A different conclusion can be drawn from a study by Corsetti *et al*, who showed that there was no difference between patients and healthy controls in identifying regular balloon inflations mixed with sham distensions, suggesting that the increased frequency of sensations often reported by IBS patients are not due to psychological response bias[116]. On the other hand, Whitehead and co-workers found that thresholds were correlated with psychological measures of anxiety and somatization[117]. However, in that study sexual abuse, reported to be associated with IBS[118], did not contribute significantly to the rectal pain sensitivity, which has been confirmed recently[119]. One explanation for these somewhat discrepant results may be that IBS patients demonstrate two different perceptual alterations: hypersensitivity to rectal distension, which is proposed to be largely due to peripheral alterations, and hypervigilance towards labeling a wide range of visceral stimuli in negatively affective terms, which is more related to psychological alterations[120].

A drawback with many of the studies assessing visceral perception in IBS is that they are made under fasting and non-stimulated conditions, under which patients normally do not report severe symptoms. However, it has been shown that repetitive sigmoid distensions - thought to imitate increased colonic motility after food intake[17], emotions[82] and stress[61] - resulted in hyperalgesia and increased viscerosomatic referral during rectal distensions in IBS patients. These findings were proposed to reflect a central sensitization of the dorsal horn neurons[121]. These data fit well with findings from studies evaluating the effects of rectal distension on spinal transmission of nociceptive signals, using a somatic cutaneomuscular flexion reflex (RIII reflex)[122], where IBS patients have an altered response[123]. Whether this is designated to changes in electrophysiological properties of the spinal nociceptive neurones or failure in the inhibition of nociceptive signals in spinal nerves is yet to be answered.

Patients with IBS often describe a correlation between their symptoms and stress, and they seem to be more susceptible to stressful events of daily life[124]. Using different methodologies, several studies now exist that demonstrate an altered visceral perceptual response in IBS patients relative to control subjects after and during stress[125-127], which might be of relevance for stress-related symptoms in IBS patients. Postprandial worsening of symptoms, as well as adverse reactions to one or more foods is common in IBS patients[128]. The effects of nutrients on colorectal sensitivity have been investigated in healthy volunteers and rectal sensitivity increases after nutrient ingestion[129]. Fat is also the main stimulus for this sensory component of the gastrocolonic response[130]. Recently we demonstrated enhancement of the colonic sensitivity in IBS patients after duodenal lipid administration as compared with healthy controls[75]. This finding has thereafter also been reproduced by other groups assessing the effect of duodenal lipid administration on rectal sensory function[131,132], making it fair to state that patients with IBS seem to have an exaggerated sensory component of the gastrocolonic response.

Visceral hypersensitivity may also be affected by motor characteristics and biomechanical properties of the gut, but studies assessing for instance rectal compliance have come to somewhat inconclusive results[108,133,134]. Moreover, a study using impedance planimetry found no clear evidence of altered biomechanical properties in the rectum in patients with IBS and hypersensitivity[135]. However, more studies assessing the relationship between sensory and motor characteristics and biomechanical properties of the gastrointestinal tract in IBS patients are needed.

To conclude, visceral hypersensitivity is currently considered to be perhaps the most important pathophysiological factor in IBS. Importantly, several external and internal factors can modulate visceral sensitivity. The link between visceral hypersensitivity and specific symptoms in IBS deserves further studies.

DYSREGULATION OF THE BRAIN-GUT AXIS

The brain-gut axis is a model describing bidirectional pathways linking emotional and cognitive centres in the brain with visceral afferent sensation and intestinal function. Several observations have led to the hypothesis of a dysfunctional brain-gut axis in the pathophysiology of IBS[136-138]. Brain imaging techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) have been used to investigate brain response during visceral stimulation with rectal distensions. Despite contradicting results, several studies indicate differences between IBS patients and healthy controls in activation of different pain processing regions such as the anterior cingulate cortex (ACC), thalamus, insula and prefrontal cortex (PFC)[139].

Using PET, Silverman et al initially reported that painful rectal distension in IBS patients resulted in reduced

ACC activity but enhanced activity in the PFC during anticipation of rectal pain suggesting a hypervigilance towards the stimulation and a failure to activate endogenous pain inhibition systems[140]. However, Mertz et al later published an fMRI study showing increased activity in the ACC indicating a normal activation pattern but a heightened sensitivity of the brain-gut axis[141]. Several brain imaging studies have been published thereafter with somewhat divergent results, with reports of increased activity in the insula, PFC and thalamus[142,143], as well as in the cingular cortex[142,144,145], but also decreased activity in the insula, amygdala and striatum[146]. The discrepancies in results are probably explained by factors such as small study populations and pooling of results from heterogeneous groups, as well as the use of different methodologies[147]. It has for instance been demonstrated that male and female IBS patients show differences in activation of brain regions[148,149], just like the activation pattern differs between IBS subgroups based on the predominant bowel habit[150]. The psychological state of the patient is also important since emotional status has been shown to modulate brain activation patterns[151]. Interestingly, changes in some brain regions have also been associated with treatment response in IBS[152]. In addition to brain imaging, IBS patients have also shown different results than healthy controls in studies using electroencephalography (EEG)[153,154] and evoked potentials[155,156].

Central nervous system communication with the gut is mediated through the parasympathetic and sympathetic pathways of the autonomic nervous system (ANS) by modulation of the enteric nervous system. Several studies have reported autonomic dysfunction in IBS[157-161]. The results are inconsistent, but increased sympathetic and decreased parasympathetic activity in IBS patients compared with healthy controls are the most frequently reported differences[162-166]. Again, the contradictory results may partly be due to varying experimental designs with differences in study populations. Anxiety and depression influences autonomic function in IBS[167], and there also seem to be discrepancies between different subgroups of IBS patients[162,165,168]. Recently Tillisch *et al* also showed gender differences in ANS response to visceral stimulus, with more prominent alterations in male IBS patients[169].

Another important system in the communication between the brain and the gut is the hypothalamic-pituitary-adrenal (HPA) axis, with important effects on GI motility, sensation and immune function[170]. Activation of this system takes place in response to both physical and psychological stressors[171]. Since patients with IBS often report stress-related symptoms[124], it has been a logical step to evaluate the HPA axis and its effect on GI function in IBS. Also here results are divergent with investigations reporting attenuated[172], normal[125] and enhanced reactivity[127,173] of the HPA-axis in IBS patients in response to various stressors. Again, different study designs, patient characteristics and other factors might be the explanation for the somewhat heterogeneous results[174].

To conclude, there appears to be alterations along the brain-gut axis and in autonomic nervous function in patients with IBS. However, there are discrepancies between existing studies, and in future studies standardization of the methodology, as well as strict inclusion criteria and evaluation of larger patient groups will enhance our understanding of the interaction between the gut and different parts of our nervous system.

CONCLUSION

The pathophysiology of IBS is complex and still incompletely known. Both central and peripheral factors, including psychosocial factors, abnormal GI motility and secretion, and visceral hypersensitivity, are thought to contribute to the symptoms of IBS. However, in order to be able to find new treatment alternatives for this big patients group more knowledge about the pathophysiology of IBS is needed.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, *Series Editor*

Evaluation of the biliary tract in patients with functional biliary symptoms

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Abstract

The aim of this paper was to describe functional biliary syndromes and methods for evaluation of the biliary tract in these patients. Functional biliary symptoms can be defined as biliary symptoms without demonstrable organic substrate. Two main syndromes exist: Gallbladder dysfunction and sphincter of Oddi dysfunction. The most important investigative tools are cholescintigraphy and endoscopic sphincter of Oddi manometry. In gallbladder dysfunction a scintigraphic gallbladder ejection fraction below 35% can select patients who will benefit from cholecystectomy. Endoscopic sphincter of Oddi manometry is considered the gold standard in sphincter of Oddi dysfunction but recent development in scintigraphic methods is about to change this. Thus, calculation of hilum-to-duodenum transit time and duodenal appearance time on cholescintigraphy have proven useful in these patients. In conclusion, ambient methods can diagnose functional biliary syndromes. However, there are still a number of issues where further knowledge is needed. Probably the next step forward will be in the area of sensory testing and impedance planimetric methods.

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Key words: Functional biliary pain; Scintigraphy; Manometry; Pain; Sphincter of Oddi; Gallbladder

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INTRODUCTION

Evaluation of the biliary tract is of importance in physiological, pathophysiological, and clinical settings. Clinical investigations in patients with well-defined, organic diseases such as gallstones, is outside the scope of this review, where focus is directed towards patients with functional biliary symptoms. Investigations aiming at morphological alterations in the biliary tract are thus not discussed in this paper.

The value of different investigative methods is often fluid: Some methods are used to render probable or document a correlation between symptoms and measurable abnormalities. Others are, or later become, real tools in the diagnostic process of patients with functional biliary symptoms. The methods can roughly be subdivided into two subgroups: Motility investigations, and methods for the evaluations of the sensory conditions. Motility comprises direct measurement of the biliary tract as well as intraluminal transport. A further evaluation of wall characteristics, e.g. by means of impedance planimetry, has, so far, only been conducted in animal experiments[1].

The present review will start with a physiologic background section, followed by a description of patients with functional biliary symptoms and evaluation methods in these patients.

PHYSIOLOGIC BACKGROUND

The function of the biliary tract is to store, concentrate, and transport bile from the liver into the duodenum. In order to accomplish this function the extrabiliary tract is composed of a ductal system, a gallbladder, and a sphincter (Sphincter of Oddi) at the termination of the common bile duct. Previously it was believed that there was an additional sphincteric structure at the junction site of the gallbladder and the cystic duct but studies have failed to prove this supposition. Smooth muscles are present in the gallbladder and in the Sphincter of Oddi (SO). Unlike previous belief, no-or only insignificant-

musculature is seen in the ducts. Active contractility is therefore only possible in the gallbladder and the SO. Bile flow into the duodenum occurs after food intake, and late in the interdigestive phase II of the duodenum[2]. The main initiator of biliary secretion-apart from bile production of the liver-is the release of the peptide cholecystokinin (CCK). This peptide relaxes the SO and contracts the gallbladder. The effect of CCK on the SO is brought about by a stimulatory action on inhibitory neurons. This effect overrides a direct stimulatory action of CCK on the SO muscles[3]. In addition to the CCK effect, a reflex relationship exists between the gallbladder and the SO, i.e. pressure increase within the gallbladder leads to relaxation of the SO (Figure 1)[4,5]. This implies that gallbladder contraction, e.g. after food intake, allows bile to run from the gallbladder into the ductal system, and further into the duodenum through the relaxed SO. Conversely, when the gallbladder is empty, the gallbladder pressure is low, and the SO actively contracts, thus allowing bile to divert into the gallbladder. A similar reflex relation is seen between the common bile duct and the SO[6]. This is important in post-cholecystectomy patients.

The gallbladder receives nerve fibres from the sympathetic and the parasympathetic systems. In addition there are a number of neurones containing peptides, e.g. CCK, vasoactive intestinal polypeptide (VIP), nitric oxide (NO), and motilin. The sympathetic nerves arise at the Th8-9 level and are relayed in the celiac plexus, with second order fibres travelling along the vascular system to the gallbladder. The parasympathetic innervation arises from both the anterior and posterior vagal trunks and reaches the gallbladder in a similar way as the sympathetic fibres, with the exception that branches from the anterior vagal nerve do not pass through the celiac plexus. Vagal activity stimulates gallbladder contraction directly, and enhances the contractile effect of CCK on the gallbladder. Sympathetic activity has the opposite effect on gallbladder motility. The sympathetic system also transmits sensory inputs to the central nervous system leading to pain. Pain is enhanced by inflammation. The ductal system and the sphincter of Oddi (SO) are innervated in a similar way as the gallbladder, with the highest neural density in the SO.

FUNCTIONAL BILIARY PAIN

Biliary pain is mainly characterized according to the symptomatic presentation of gallstone attacks. As in many other gastrointestinal disorders this is not simple, as a variation in symptomatic presentations can be seen in some patients[7]. Nevertheless, according to the existing literature, and the experience of experts, functional biliary pain was defined as absence of demonstrable organic substrate, and[8]: Episodes of severe steady pain located in the epigastrium and right upper quadrant. A number of additional criteria are outlined. One of these is that the pain attack should last at least 30 min. This seems substantiated in patients where the symptoms are derived from the gallbladder. However, if symptoms originate in the SO, this criterion will still await validation. Two types of functional biliary pain syndromes have been described: Gallbladder dysfunction and sphincter of Oddi dysfunction (SOD). The symptomatic presentation seems rather similar, but obviously patients with gallbladder dysfunction still have the gallbladder in situ, whereas SOD dysfunction most often-albeit not unequivocally-is seen in post-cholecystectomy patients.

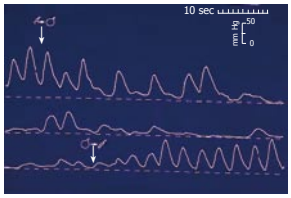


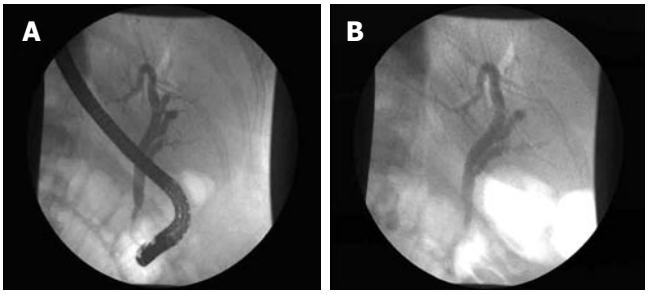
Figure 1 Sphincter of Oddi manometry in a dog during gallbladder distension (upper tracing), and gallbladder emptying (lower tracing). Distension of the gallbladder-mimicking gallbladder contraction -leads to relaxation of the sphincter of Oddi thus permitting free flow of bile into the duodenum. (After reference 5 with permission).

Gallbladder dysfunction

Gallbladder dysfunction denominates a condition where the gallbladder empties insufficiently in a patient with biliary symptoms, without demonstrable organic substrate, such as gallstones. The prevalence of biliary-type pain without gallstones was found to be 2.4%[9]. Other population studies have found prevalences as high as 7.6% in men[10] and 20.3% in women[11]. Per definition patients with gallbladder dysfunction have impaired gallbladder emptying. Later in this paper the methodology is described. The mechanism of pain is rather obscure: Generally it is believed that some kind of functional obstruction at the transition zone between the gallbladder and the cystic duct exists, leading to gallbladder pressure increase. Possibly, a combination with inflammation amplifies sensory firing. Primary hypersensitivity can also not be ruled out and central neuroplastic changes may be responsible for visceral hypersensitivity. Nevertheless, in a randomised controlled investigation[12], it was shown that patients with gallbladder dysfunction would benefit from cholecystectomy. A number of other papers have supported this finding[13,14]. However, there are still a number of critical issues to be addressed[15].

Sphincter of Oddi dysfunction

Sphincter of Oddi dysfunction (SOD) denominates patients with biliary-type pain and dysfunction of the Sphincter of Oddi. The condition is most often seen in post-cholecystectomy patients. The prevalence of symptoms suggesting SO dysfunction is about 1.5% after cholecystectomy, being more frequent in women[16]. SO dysfunction can be subdivided into three groups according to clinical presentation, laboratory results, and ERCP findings[17]: (1) Patients with type I SO dysfunction have biliary-like pain, elevated liver function tests documented on two or more occasions, delayed contrast drainage (more than 45 min, (Figure 2), and a common bile duct diameter equal to or greater than 12 mm at ERCP; (2) Type II patients present with biliary-like pain, and only one or two of the above mentioned criteria; (3) Type III patients only have biliary-like pain, and none of the above mentioned criteria. The mechanism of pain is also rather obscure in this group of patients. From a theoretical point of view SO abnormalities could give rise to pain due to impeded flow, ischemia arising from spastic contractions, and hypersensitivity of the papilla or the common bile duct.



During ERCP (time 0) 45 min after ERCP

Figure 2 Endoscopic cholangiogram during investigation, and 45 min after contrast injection in a patient with complete contrast retention.

These mechanisms may solely or in combination explain the genesis of pain[18]. In a prospective randomised study it was shown that pain could be alleviated by endoscopic sphincterotomy[17].

EVALUATION METHODS

Quantitative hepatobiliary scintigraphy

Quantitative hepatobiliary scintigraphy (QHBS) is a non-invasive scintigraphic method where it is possible not only to assess the patency of the biliary system but also to measure the rate of bile flow (Figure 3). 99mTc-N-2,6-dimethylphenylcarbamoylmethyliminodiacetic acid (99mTc-EHIDA) and numerous of its analogues, such as 2, 6-diisopropyliminodiacetic acid (DISIDA) and 2, 4, 6-trimethyl-3-bromoiminodiacetic acid (mebrofenin) are the tracers most widely used for hepatobiliary imaging. After (iv) injection, these 99mTc agents bind loosely to serum proteins and are then rapidly taken up by the hepatocytes and excreted into the bile. This uptake mechanism is similar to that of bilirubin, and hyperbilirubinaemia therefore has an inhibitory effect. After excretion by the hepatocytes, the bile continues to flow into the bile canaliculi, the bile ducts and the gallbladder, and then empties into the duodenum through the SO.

Patients should be fasted for a minimum of 4 h prior to the investigation. The gamma-camera is positioned over the right upper abdomen of the supine patient. After an iv bolus injection of 140-180 MBq 99mTc-IDA derivative, gamma-camera images are acquired on a computer (1 frame/min) for 90-120 min. If needed, analogue images can also be obtained every 10 min for the first hour, with delayed images if required (first image 500 000 counts, and then subsequent images for the same length of time). The analogue pictures should be examined for liver radiotracer uptake, size, shape and homogeneity. Under normal circumstances the liver uptake of 99mTc-IDA compounds is rapid, with little if any tracer in the blood pool by 5 min. In most normal fasting subjects, radioactivity appears in the gallbladder (GB) and in the small bowel by 1 h after its administration. The extent of visualisation of the hepatic ducts is variable but the left duct tends to be more prominent than the right. In patients without extrahepatic

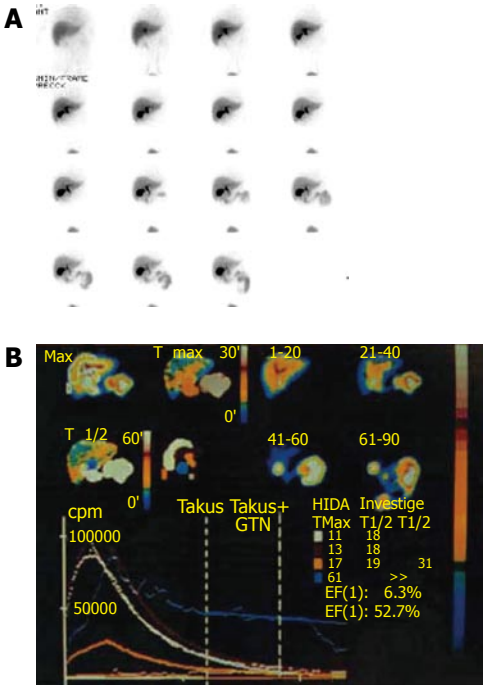


Figure 3 Hepatobiliary scintigraphy. **A:** Pictures at different time windows; **B:** Time activity curves are shown for different regions of interest.

bile duct obstruction, the level of radioactivity of the common bile duct (CBD) reaches its maximum before 45 min. Excretion of radioactivity into the duodenum occurs by 60 min in 81% of patients with the GB *in situ*. In the remaining 19% of patients without bowel activity at 60 min, duodenal visualisation is always present after exogenous CCK administration[19].

Gallbladder phase imaging

The GB contraction after consumption of a meal or administration of an exogenous CCK analogue (CCK octapeptide or caerulein) can also be measured by means of QHBS. These data can be acquired in a separate study between 60 and 90 min after the initial isotope administration. QHBS combined with administration of a meal or an exogenous CCK analogue is a validated and accurate method for the measurement of GB motility, with satisfactory reproducibility of both parametric variables and emptying patterns. The method of measuring GB is to place a region of interest (ROI) over the GB with a ROI over the liver background (adjacent to the GB), then to generate a time-activity curve for the GB and to calculate the GB ejection fraction (EF) via the following formula: EF% = (GBmax-GBmin)/GBmax × 100. The degree of GB emptying is CCK dose-dependent, and therefore the mode of administration has a critical effect on the net GB contraction. The GB EF increases as the dose of CCK is increased from 0.5 to 3.3 ng/kg per minute. Then begins to decrease when larger doses are infused, especially when CCK is given as a rapid iv bolus. A large non-physiological dose produces a low EF in association with abdominal pain, even in normal subjects. It has become clear that the GB EFs obtained depend on the procedure used but a

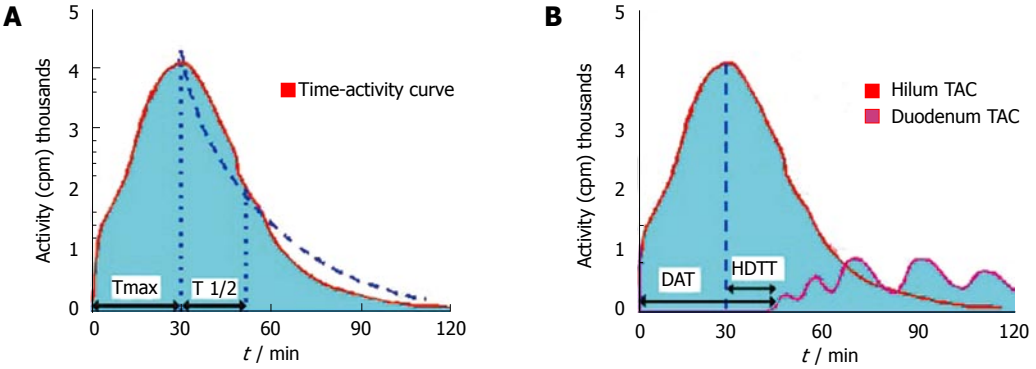


Figure 4 Hepatobiliary scintigraphic activity curves of regions of interest in sphincter of Oddi dysfunction.

GB EF over 35%[12,20,21] can be regarded as indicative of a normal GB function if it is evoked by a total infusion dose of 10 ng/kg CCK, administered during 3-10 min.

Scintigraphic features of gallbladder dysfunction

In clinical practice, the distinctive feature of a GB dysfunction is impaired GB emptying. A GB EF under 35% is a sensitive test for the identification of patients with chronic cholecystitis or GB dysfunction[12,20,21]. However, there is a considerable overlap in EF values[15]. Many of the patients experience a reproduction of biliary pain during CCK infusion. Administration of nitro-glycerine before CCK infusion can abolish biliary pain and normalise the GBEF in a subset of patients with gallbladder dysfunction.

Scintigraphic features of sphincter of Oddi dysfunction

The scintigraphic signs in SOD depend upon the presence or absence of a GB in situ, albeit the latter is rare. In patients with SOD and GB in situ, an intact GB serves as a low-pressure reservoir by compensating the increased bile flow that is diverted into it by the tight and hypertonic SO. Therefore, the CBD does not dilate and the rate of emptying of the isotope from the CBD is usually in the normal range. After CCK administration to patients with a functional SOD, the SO relaxes (except in SOD patients with a paradoxical response to CCK, which is a relatively rare phenomenon) and, if the GB function is intact, the GB EF remains normal. In cholecystectomized subjects with a normal liver function, the flow of the bile from the liver to the duodenum is mainly regulated by the motor activity of the SO. HIDA compounds, which are taken up and secreted by the liver into the bile, can therefore be used as markers to measure the rate of bile flow and indirectly to assess the patency of the SO. In asymptomatic cholecystectomized patients with a normal SO function, HIDA excretion and transit from the liver into the duodenum is rapid, and the time-activity curves are exponential, exhibiting only minor patient-to-patient variations[19]. In cholecystectomized patients with SOD however, cholescintigraphy reveals a prominent bile stasis within the intra- and extrahepatic bile ducts, and a marked delay in the transpapillary isotope transit[22-24]. Retention of a 99mTc-IDA tracer after 120 min in the visually prominent bile ducts on static images of the planar HBS was noted to be a good predictor of an abnormal biliary drainage. Quantitative cholescintigraphic parameters may afford valuable numerical information on the bile flow impairment in the presence of a SOD, thereby increasing the sensitivity (Figure 4). Unfortunately, it is not clear, which scintigraphic variable is optimal to express SOD. We recently compared the sensitivities of these parameters in cholecystectomized patients with SOD diagnosed by manometry, which showed that HDTT was the best predictor of SOD, followed by DAT, and then the $T_{1/2}$ parameters of the hepatic hilum and CBD Madacsy). The quantitative parameters of the CBD seem to be relatively insensitive, which is probably due to the disturbing influence of other factors, such as the CBD diameter and the duodenal motility phase during the study. In fact, we found that the SO basal pressure relative to the CBD pressure exhibited the best correlation with the QHBS parameters. CCK administration increases the hepatic bile secretion and relaxes the SO; it therefore washes out the bile stasis from the ducts if there is no anatomic obstruction. A differentiation between a fixed anatomic obstruction (organic SO stenosis, bile duct stricture or stones, *etc.*) and a functional obstruction (SO spasm) can also be made by amyl nitrate or nitro-glycerine administration during QHBS. In order to measure SO relaxation after the administration of a smooth muscle relaxant in patients with SOD, after the baseline evaluation of the QHBS parameters, at 60 min amyl nitrate was administered and the $T_{1/2}$ parameter of the CBD was recalculated. A reversible biliary obstruction on the QHBS (an obstructive pattern relieved after amyl nitrate) indicates a functional SOD, i.e. an SO spasm, whereas a persistent accumulation of the isotope in the biliary tree (during the baseline condition and after amyl nitrate) suggests a fixed organic obstruction[25]. A differentiation between stenosis and dyskinesia of the SO may be important from a clinical point of view, and it might modify the therapeutic strategy, since endoscopic sphincterotomy is of great benefit in patients with a persistently elevated basal SO pressure and SO stenosis. To summarise, QHBS may be regarded as a useful method for study of the bile flow dynamics in patients with postcholecystectomy syndrome and suspected SOD. QHBS may be applied not only as a simple and non-invasive screening test before SO manometry but also as a reliable diagnostic method during the evaluation and follow-up of patients with suspected SOD.

Ultrasonography

US has become a widely used method for visualisation

of the abdominal organs. As it is a non-invasive method without radiation exposure, it has also become a popular method for the investigation of motility disorders of the GB and the SO. US methods are relatively cheap and widely available and they also afford excellent reproducibility and sensitivity. However, their diagnostic accuracy is appreciably investigator-dependent and also influenced by the different forms of methodology.

Ultrasonographic measurement of GB contraction

The GB contraction induced by exogenous CCK or CCK analogue (caerulein) administration can be measured by means of either US or QHBS, but methods involving QHBS are currently regarded as the gold standard. QHBS offers the advantage over US of allowing simultaneous visualisation of the bile ducts, together with non-geometrical measurement of the GB contraction. However, the lack of radiation exposure is a favourable feature of the US methods, because it can be repeated several times in the same patient, which is extremely important in motility studies if normal physiology is investigated. US is also able to determine the approximate volume of the GB, which may be of some importance in the pathophysiology of GB motility. Different formulae are known for calculation of the GB volume. The most widely used is the sum of cylinders method, or Dodd's formula. With this method, the GB volume (GBV) can be calculated if one multiplies the three axes together and then by Pi/6[26]. The GB EF is calculated from the fasting and post-stimulation GBVs via the well-known formula: GBEF (%) = GBVmax - GBVmin/GBVmax. The refilling of the GB is another characteristic feature of GB motility, which can be calculated as a percentage of the ratio between the GBV in the most contracted state and at the maximum after refilling. The two main types of stimuli, which can be used to evoke GB contraction, are food intake and administration of CCK or a CCK analogue.

Ultrasonographic bile duct imaging

Bile duct imaging with US is more difficult than visualisation of the GB. Most investigators use the right anterior oblique view to visualise the common hepatic duct within the porta hepatis, where the surrounding liver parenchyma gives a good acoustic window. Visualisation of the pancreatic duct in the body of the pancreas may be problematic in a number of patients, because the presence of gas in the stomach and in the intestines above it means that an acoustic window is not available. Moreover, exact determination of the pancreatic duct diameter necessitates its measurement in two different part of the pancreas. The upper limit of normal diameter of the common hepatic duct (also termed hepatocholedochus, because it is not possible to differentiate between common hepatic duct and CBD by US) is less than 8-10 mm, and of the pancreatic duct is less than 2-3 mm.

Ultrasonographic assessment of sphincter of Oddi dysfunction

Ultrasonography has been reported as a method for diagnosing SO dysfunction. The authors measured the bile duct diameter before and after CCK infusion or ingestion of a fatty meal[27,28]. However, these methods have not gained acceptance, probably because of low reproducibility.

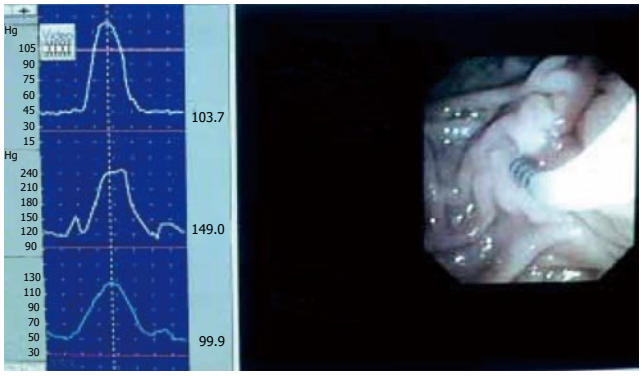


Figure 5 Combined video- and manometric recording in the sphincter of Oddi during endoscopy.

Sphincter of Oddi manometry

SO manometry can be done during endoscopic retrograde cholangio-pancreatographic (ERCP) investigation. A triple lumen catheter with sideholes spaced 2 mm apart is introduced into the common bile duct, and then withdrawn until positioned in the SO segment. The catheter is slowly perfused from a hydraulic capillary infusion pump, and external transducers are connected with a computer for direct reading of the manometric tracing. Additionally the readings are stored for later analysis[29,30]. The SO is a high-pressure zone with an elevated baseline and superimposed phasic contractions. The readings may suffer from periodic superimposed artefact patterns. Usually these are easily recognised, but simultaneous endoscopic videorecording may be helpful in separating SO activity from artefact phenomenon (Figure 5)[31]. The parameters measured are baseline pressure and phasic wave amplitude, duration, frequency, and propagation mode. Baseline pressure is measured with the duodenal pressure as zero reference. The amplitude is measured with baseline pressure as zero reference. When evaluating the propagation modality, proximal and distal readings are compared.

Normal SO manometry

In healthy subjects the baseline pressure is around 5-15 mmHg, phasic wave amplitude 100-140 mmHg, wave duration 4-6 s, and frequency 3-6 waves per minute. Most contractions are coordinated, and the direction of contractions is mostly antegrade, with only 5%-20% being retrograde[32]. After cholecystectomy no significant alterations are seen in patients who no longer have symptoms[33,34].

Abnormal SO manometry

SO manometric abnormalities comprise elevated baseline pressure, increased phasic wave amplitude, frequency, and disordered propagation mode (Figure 6, Figure 7). It has been suggested to define SO abnormality as a baseline

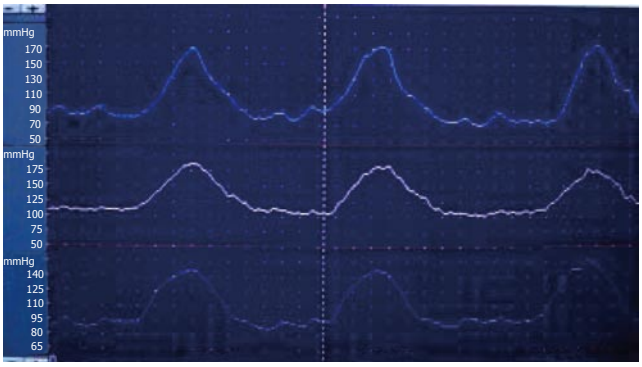


Figure 6 Endoscopic sphincter of Oddi manometry in a patient with elevated baseline pressure.

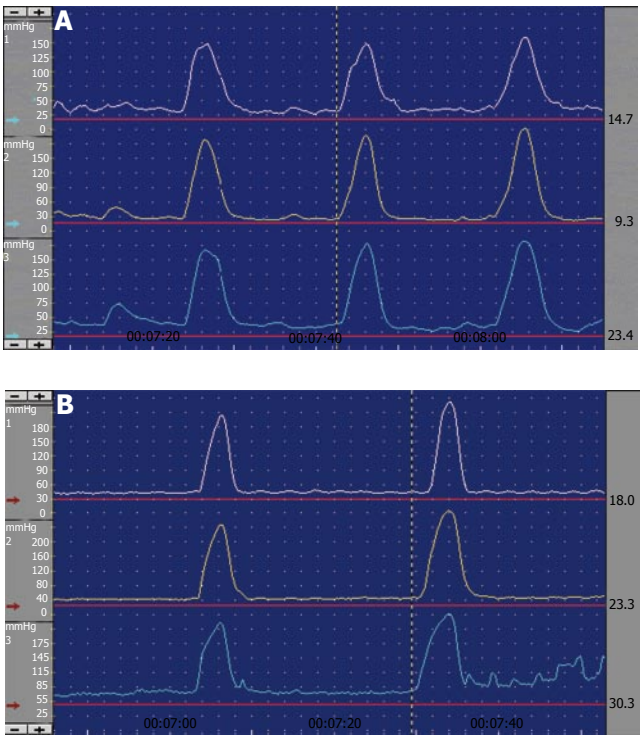


Figure 7 Endoscopic sphincter of Oddi manometry. **A**: Normal antegrade contractions; **B**: Abnormal retrograde waves.

pressure of more than 40 mmHg, phasic amplitude above 350 mmHg, frequency of more than 7 waves per minute; and a pattern of more than 50% retrograde waves[8]. The clinical importance of these abnormalities is not clear, except for elevated baseline pressure, where it was shown in a randomised study that patients with this abnormality improved clinically after endoscopic sphincterotomy[17].

Pain studies

The fact that there are still "holes" in our understanding of patients with functional biliary pain has led investigators to focus on sensory mechanisms. Clinically, it has been observed that some patients develop pain upon manipulation with the papilla (tender papilla), or during intraductal contrast injection. A further step forward was taken with the demonstration of duodenal-specific visceral hyperalgesia in post-cholecystectomy patients with persistent abdominal pain[35]. Stawowy *et al*[36-38] has investigated the referred pain area in patients with uncomplicated gallstones and acute cholecystitis before and after cholecystectomy. Although hypersensitivity was found in both groups of patients, the hypothesized prolonged neuroplastic changes, as a possible explanation of post-cholecystectomy syndrome could not be proven. However, in the future it will be of importance to conduct studies in patients with post-cholecystectomy syndrome and also in patients with suspected gallbladder dysfunction.

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TOPIC HIGHLIGHT

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Upper gastrointestinal sensory-motor dysfunction in diabetes mellitus

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Abstract

Gastrointestinal (GI) sensory-motor abnormalities are common in patients with diabetes mellitus and may involve any part of the GI tract. Abnormalities are frequently sub-clinical, and fortunately only rarely do severe and life-threatening problems occur. The pathogenesis of abnormal upper GI sensory-motor function in diabetes is incompletely understood and is most likely multi-factorial of origin. Diabetic autonomic neuropathy as well as acute suboptimal control of diabetes has been shown to impair GI motor and sensory function. Morphological and biomechanical remodeling of the GI wall develops during the duration of diabetes, and may contribute to motor and sensory dysfunction. In this review sensory and motility disorders of the upper GI tract in diabetes is discussed; and the morphological changes and biomechanical remodeling related to the sensory-motor dysfunction is also addressed.

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Key words: Diabetes; Esophagus; Stomach; Intestine; Motility; Pain; Diabetic neuropathy; Hyperglycemia; Remodeling

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disease requiring lifelong medical attention in order to limit the development of potentially devastating late complications and to manage them if they occur. In the USA, the per capita cost of healthcare in 2002 was $13 243 for patients with diabetes and $2560 for patients without diabetes[1]. Gastrointestinal (GI) disorders are common in diabetic patients[2,3]. As many as 75% of patients attending DM clinics report significant GI symptoms[2]. The entire GI tract from the esophagus to the anorectal region may be affected. Common complaints include dysphasia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting and diarrhea. The symptoms may be severe and substantially decrease quality of life. The pathogenesis of the GI abnormalities is complex of nature, multi-factorial (motor dysfunction, autonomic neuropathy, glycemic control, psychological factors, *etc*.) and is not well understood[4]. A number of abnormal conditions have been described in different segments of the GI tract in patients with diabetic autonomic neuropathy (DAN): esophagus (dysmotility), stomach (dysmotility, delayed emptying) and small and large bowel (dysmotility, delayed transit, bacterial overgrowth and diarrhea)[4]. Only a few studies have addressed the visceral sensory function in DM[5-7] and have demonstrated abnormalities in perception thresholds, vagal tonus and evoked brain potentials in patients with DAN. This indicates that DM related neuronal changes may be located both in the peripheral and in the central nervous system (CNS). As mentioned the entire GI tract may be involved in DM, but our review describes the upper GI tract only.

Many studies have demonstrated prominent morphological changes of the small intestine and esophagus in DM[8-11]. Lately, several studies have described biomechanical remodeling as well as morphological remodeling in experimental diabetic rats[12-18]. Recently, Frøkjær *et al*[19] have shown that both the neuronal function of the contractile system as well as the structural apparatus of the GI tract may be affected in patients with longstanding DM and DAN. Therefore we suggest that

along with DAN and glycemic control, the structural and biomechanical changes may play important roles in the symptomatology of GI abnormalities in long-standing DM. The future management of diabetic patients with GI symptoms may develop accordingly.

SYMPTOMS FROM THE UPPER GI TRACT IN DIABETES MELLITUS

Motor and sensory abnormalities in DM may affect the entire GI tract or part hereof, and the perceived symptoms may originate from one or several parts of the gut, such as the esophagus, stomach and small intestine[20,21]. The prevalence of upper GI symptoms is high in both insulin dependent DM (IDDM) and non-insulin dependent DM (NIDDM)[22-28]. The symptoms relating to the esophagus, stomach and small intestine are as follows: (1) Esophagus: Heartburn, dysphagia and chest pain[37]; (2) Stomach: weight loss and abdominal pain[38]; (3) Small intestine: Diarrhea, discomfort, pain and pseudo-obstruction. Most symptoms are non-specific of nature and may relate to other GI disorders not necessarily related to DM. Therefore, when dealing with GI symptoms, some specific issues need to be addressed. Chest pain may relate to reflux or esophageal motor disorders, but ischemic heart disease and other causes of non-cardiac chest pain are also possible causes and must be excluded. Dysphagia, the most characteristic symptom of impaired esophageal transit, may be caused by motility disorders of the esophagus. It is very important, however, to emphasize that dysphagia is more often caused by mechanical obstruction such as tumors and peptic stenosis than by motility disorders. A number of other incidental conditions must be excluded when diabetic gastroparesis is suspected: gastric outlet obstruction caused by tumors and ulcer disease; metabolic abnormalities such as diabetic ketoacidosis or uremia and side effects of pharmacotherapy. A gastroscopy and biochemical screening followed by physical examination are obligatory requisites to reach the correct diagnosis. The symptoms of diabetic gastroparesis tend to increase in intensity and frequency over the duration of diabetes in patients affected. Most often symptoms are vague and nonspecific such as early satiety, slight abdominal discomfort and perhaps bloating, and fortunately more rarely do nausea and vomiting develop. When nausea and vomiting becomes continuous hospitalization is needed to control glucose homeostasis, electrolytes and fluid substitution is needed. This is a potential life threatening situation. In the long run diabetic gastroparesis may be accompanied by poor diabetic control, weight loss and diminished quality of life partly due to frequent hospital contacts. The symptoms may last for days to months, and do often occur in cycles with symptom free intervals[29,30]. Diabetic patients frequently report abdominal pain and this may be the only symptom of diabetic gastroparesis; however, abdominal pain can also be seen in diabetic ketoacidosis and severe metabolic acidosis[31]. These conditions are followed by other symptoms as well and therefore distinguishable from gastroparesis. Diabetic patients with thoracic polyradiculopathy, a rare condition, may also suffer from abdominal pain[32]. Diarrhea in DM patients may be induced by a number of factors. These may include food composition, abnormal intestinal motility, small intestinal bacterial overgrowth, excessive loss of bile acids, pancreatic insufficiency and more[33]. Abnormal small intestinal motility (rapid or delayed transit) is a frequent condition in the diabetic patients as described below. Rapid transit may induce an increase in intra-luminal contents that reach the caecum, whereas delayed transit may cause bacterial overgrowth, both potentially resulting in diabetic diarrhea. Bacterial overgrowth has been reported in up to 40% of diabetic patients with diarrhea[34,35]. Celiac disease is overrepresented in IDDM and a cause of severe diarrhea to be excluded when dealing with diabetic diarrhea[36]. In its most fulminate form, diabetic diarrhea is a devastating and horrible condition for the person affected, sometimes resulting in catastrophical nightly soiling in bed and an uncontrollable condition during daytime. Most often, fortunately, this condition is self limiting and symptoms are more manageable.

SENSORY DYSFUNCTION

Although both the afferent and efferent nerves are affected in DM, the data related to the sensory dysfunction of the GI tract are sparse compared with those relating to the motor dysfunction of the upper GI tract. Elevation of perception thresholds to esophageal electrical stimulation has been observed in patients with DAN and different severity of GI symptoms[7]. Increased vagal tonus and abnormal evoked brain potentials to mechanical and electrical stimulation of the esophagus has also been shown[5,6]. Rayner *et al* performed isovolumetric and isobaric distensions of the proximal stomach in ten randomly selected patients with IDDM[39]. They demonstrated that the perception of gastric distension during euglycemia was increased compared with healthy controls. To study mechanisms behind postprandial symptoms in patients with diabetes, the gastric accommodation of the meal was assessed by abdominal ultrasound[40]. In DM patients, a large proximal stomach was associated with perception of fullness and a large antrum was associated with perception of pain after a meal. More recently, Frøkjær *et al*[41] used a multimodal stimulation device (Figure 1) to investigate the visceral sensitivity to mechanical, thermal and electrical stimulation in the esophagus and duodenum in IDDM patients with DAN and GI symptoms. This study demonstrated that the patients had decreased sensitivity to the stimulations of the esophagus and duodenum. This indicates that the affection of the sensory nerves is widespread in the GI tract. As the multimodal approach is thought to stimulate the mucosa, submucosa and muscle layers differentially, the disease seems to be generalized to nerves in all layers of the gut.

MOTILITY DISORDERS

Long ago, it was known that abnormal motility of the GI tract occurred during the development of DM[42]. So far many studies have demonstrated that DM patients have slow transit and abnormal motility. The most frequent

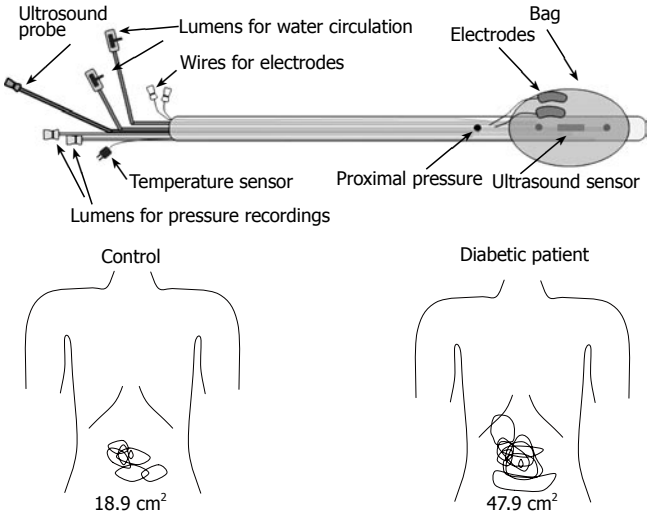

Figure 1 Top: The probe design allowing mechanical, heat and electrical stimulation of both the oesophagus and duodenum. The centre of the bag contains the sensor of the ultrasound probe imaging the oesophagus and duodenum during mechanical distension. The thermal stimuli were given by a pump system, which re-circulates water at 60 ℃ through two channels in the probe. The electrodes allowed electrical stimulation delivered directly to the mucosal surface. Bottom: The somatic referred pain areas to mechanical distension of the duodenum for the controls (left) and diabetic patients (right). The areas were larger for the diabetic patients.

Table 1 Motility disorders of upper GI tract in DM

Organ	Motility disorder
Esophagus	Amplitude[88,89] and number[46,90] of peristaltic contractions↑
	Number of spontaneous and non-propagated contractions↑[45]
	Amplitude of lower esophageal sphincter pressure↓[88]
	Multi-peaked contractions[78,79]
Stomach	Antral IMMC↓[87]
	Post-prandial antral activity and the number of antral contractions↓[91]
	Pyloric dysmotility[92]
Small intestine	Frequency and amplitude of the antropyloroduodenal contractions↑↓[93]
	Duration of MMC cycle↑[85]
	Early recurrence of the MMC and clusters of contractile activity[94]

motility disorders of upper GI tract are shown in Table 1.

Upper GI transit disorders in DM

Esophagus: Impaired esophageal transit has been reported both in IDDM and NIDDM patients[43-47]. The esophageal transit appears to be delayed in about 50% of patients with long-standing DM[48]. The retarded esophageal transit in the DM usually reflects either peristaltic failure or focal low-amplitude pressure waves[49].

Stomach: Several animal studies reported a slowing gastric emptying in IDDM and NIDDM rats[50-55], whereas other studies on IDDM and NIDDM animals demonstrated that gastric emptying increased[56-59]. Using radionuclide measuring techniques it has been demonstrated that gastric empting of solid, or liquid meals was abnormally slow in 30%-50% of patients with long-standing IDDM and NIDDM[60,61]. The gastroparesis in DM has been known clinically for more than 50 years[29]. It is not surprising that the gastric emptying delay in DM is related to both slow transit with increased retention of food in the proximal and distal stomach[62,63], and abnormal motility of the gastric wall[64].

Small intestine: Delayed and rapid transit in the small intestine was observed in animal diabetic models[65,66,67,68]. El-Salhy reported that the GI transit was rapid in non-obese diabetic mice[67] and was slower in obese diabetic mice[68]. Anjaneyulu and Ramarao[69] reported an increase in intestinal transit and a decrease in intestinal tone due to increased cholinergic and decreased beta-adrenergic receptor activities in DM rats. Slow small intestinal transit in DM patients have been documented using breath hydrogen appearance time after the ingestion of lactulose[70,71], by using radiopaque markers[72,73] and by use of metal-detector test[74]. On the other hand, Keshavarzian and Iber[75] investigated intestinal transit in IDDM patients after the ingestion of both liquid and solid meals and showed abnormal fast intestinal transit in their sample of diabetic patients. Nguyen *et al*[76] used intraluminal multiple impedance measurements to identify the postprandial duodenal chyme transport in patients with long standing IDDM. They demonstrated that the patients had disturbed propulsive chyme transport through the duodenum and the duodenal chyme clearance activity was decreased.

The transit disorders can occur in any region of the gut and in every stage of diabetes[74], and can affect each other. Since transit of food through the esophagus is relative fast, the gastric emptying rate is the major determinant of the food delivery to the small intestine. The relationship between esophageal transit and the rate of gastric empting appears to be poor[43] and the gastroparesis is often associated with the intestinal transit delay in DM[74,77].

Abnormal patterns of upper GI motility in DM

Esophagus: Esophageal manometric abnormalities occur in over 50% of patients with DM (see Table 1)[37]. The reduced amplitude of lower esophageal sphincter pressure is in accordance with the increased prevalence of gastro-esophageal reflux disease in DM. More recently the evoked esophageal contractile activity to standardized bag distension was assessed using a specialized ultrasound-based probe by Frøkjær *et al*[19]. A balloon-like bag was positioned 10 cm above the lower esophageal sphincter and inflated. It was demonstrated, both at the bag and 6 cm proximally, that the distension induced hyperreactivity and impaired the coordination of the contractions in the diabetic patients.

Stomach: Motility disorders of the fundus and pylorus have been demonstrated in the diabetic animals[80-83]. In the human studies, it is recognized that disordered gastric contractile activity as assessed by manometry and gastric emptying occurs frequently in DM[84,85]. The motility disorders may include three aspects: Inter-digestive migrating motor complex (IMMC), amplitude and frequency of contractions, and pyloric dysfunction (Table 1).

Small intestine: Camilleri and Malagelada reported that

small intestinal motility was abnormal in about 80% patients of long-standing DM with delayed gastric emptying[86]. Both postprandial and fasting small intestinal dysmotility in the DM was reported (Table 1). Dooley *et al*[87] studied fasting GI motility by manometry for a mean of 210 min in a group of 12 NIDDM patients with diarrhea and DAN. The patients showed grossly disordered motility. The migrating motor complex (MMC) disorders reflect the prolongation of phase II without change in the duration of phase I and III. The results of studies on postprandial motility at the level of the small intestine are inconsistent. However, abnormal motility patterns were observed in the diabetic subjects[85].

Table 2 Acute hyperglycemia and upper GI disorders

Motility disorder	Sensory disorder
Peristaltic wave duration[98]↑	Amplitude of the cortical evoked brain potentials to moderate esophageal distension↑[98]
Peristaltic velocity in the distal part of the esophagus↓[103]	Perception during proximal gastric distension in healthy subjects both in the fasted state and during intra-duodenal lipid infusion↑[95]
Pressure of the low esophageal sphincter↓[103]	Influencing the postprandial fullness in IDDM patients↑[105]
Gastric emptying of both solids and nutrient liquids↓[104]	
Motility index and propagation of duodenal and jejunal waves↓[84]	
Cycle length of inter-digestive motor activity in the fasted state↓[56]	
Small intestinal transit↓[71]	
Proximal duodenal pressure waves↓[104]	

PATHOPHYSIOLOGY OF GI MOTILITY AND SENSORY DYSFUNCTION IN DM

Hyperglycemia

Disordered GI function in DM has been attributed to irreversible DAN but it is now clear that acute high blood glucose concentration *per se* have a major reversible influence on upper-GI tract motility and sensory function. An animal study demonstrated that the correction of hyperglycemia to euglycemic levels restores the delayed transit[65]. Several studies in both healthy subjects and DM patients have shown that the GI motor function is impaired during acute hyperglycemia (Table 2)[84,95-97]. Marked acute hyperglycemia affects the motility in every region of the GI tract[98]. This may indicate that cholinergic activity is affected during hyperglycemia. Hyperglycemia may also affect the perception of sensations arising from the GI tract (Table 2). However, much of the data have been observational, and there is relatively little information relating to potential mechanisms by which these effects are mediated. Because both stimulatory and inhibitory effects occur during the hyperglycemia, the effects of glucose are likely to be mediated by neural or humoral mechanisms, rather than a direct effect on the smooth muscle of the GI tract. The secretion of pancreatic polypeptide, which is under vagal cholinergic control, is diminished during acute hyperglycemia in healthy control subjects[99]. In healthy volunteers, plasma concentrations of motilin are less during hyperglycemia when compared with euglycemia[100]. Considering the effects of systemic changes in blood glucose concentrations, animal studies have revealed the presence of glucose-responsive neurons in the central nervous system, which may modify vagal efferent activity[101]. Neurons responsive to glucose have recently been identified in the rat small intestine[102], but their response to systemic rather than luminal glucose is unclear. Much work is required to elucidate the neural, humoral, and cellular mechanisms by which systemic glucose levels affects GI motility and sensation.

Peripheral and central neuronal changes

DAN is seen as a major factor in the pathogenesis of disordered GI motor and sensory functions in DM[106,107]. Although GI manifestations related to DAN are diverse, many GI complications of DM seem to be related to DAN[19,48,72,108-110].

Histological findings: The best-characterized signs of damage to the autonomic nervous system during DM are morphological animal studies[111-117]. The number of myelinated axons in the vagosympathetic trunk is decreased in diabetic rats[118]. In the GI tract, many changes of nerves and ganglia were observed i.e. (1) dystrophic axonopathy[119]; (2) degeneration of mesenteric nerves and ganglia[111]; (3) number of vasoactive intestinal peptide (VIP)-IR neurons in myenteric ganglia[112]; (4) relative volume density in myenteric plexus[120]; (5) number of adrenergic and serotonergic neurons[111]. In addition, the surrounding tissue is also often disturbed. There is a thickening of the endothelium[121,122], which may sensitize axons to damage from increased pressure or decreased oxygen and glucose availability. It is thus possible that axonal damage may be secondary to disorders in tissue surrounding the neurons. Nitric oxide (NO) is a key neurotransmitter in the regulation of GI motor function[123,124]. In rodents with streptozotocin (STZ)-induced diabetes, NO synthase expression in the gastric myenteric neurons is diminished[125] and associated with delayed gastric emptying. The results of morphologic studies of the vagal nerves in diabetic patients are less consistent. One study demonstrated a decrease in unmyelinated axons in the abdominal vagus, another study found no abnormalities[126,127].

Clinical findings: One study has shown that abnormal esophageal motility is more frequent in the diabetic patients with evidence of DAN than in those without[37]. The presence of abnormal gastroesophageal reflux in diabetic patients has also been associated with the existence of DAN[128]. Increased cholinergic tone may relate to multipeaked waves in the diabetic esophagus in these patients[78]. Gastric emptying largely depends on vagus nerve function, which can be severely disrupted in DM[60]. Studies using cardiac autonomic nerve function tests to assess the involvement of the autonomic nerve system in diabetic patients have indicated that the prevalence and severity of dysmotility of the small intestine is substantially greater in DM patients with DAN compared to patients with normal autonomic function[129]. Diarrhea is evident in 20% of diabetic patients, particularly those with known DAN[130,131]. Altogether, abnormal upper GI motility in DM has been associated to DAN. However, the question arises as to whether neuropathic changes in the intramural GI plex-

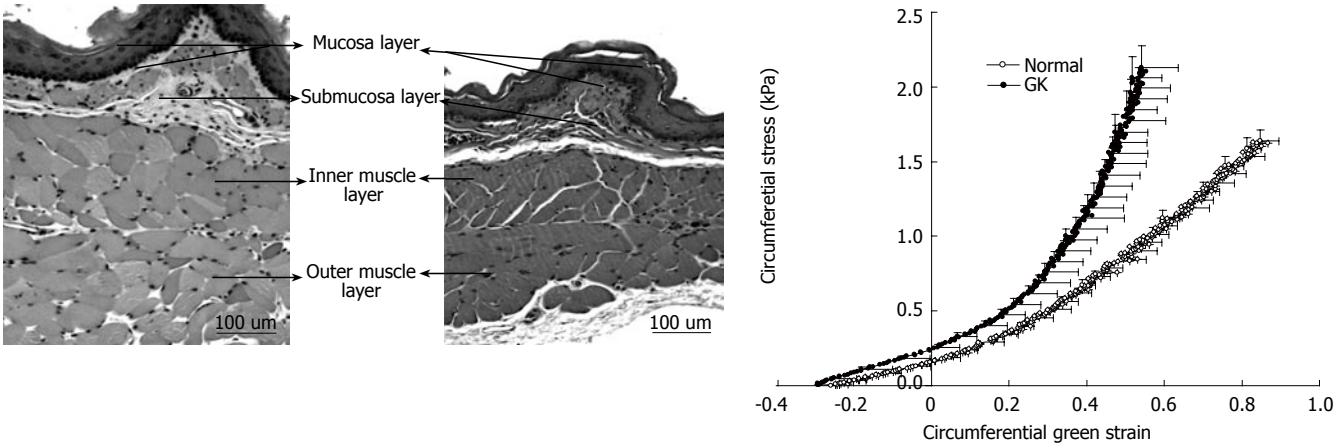

Figure 2 Microphotos of esophageal layer thickness in GK rats (left) and normal rats (middle). The muscle layer was biggest in the GK esophagus. Correspondingly the circumferential stress-strain curves of diabetic and non-diabetic esophageal wall showed in the right. The curve in the GK rats was shifted to the left, indicating that the esophageal wall stiffness increased.

uses, the extrinsic neuronal pathways or in the central nervous system are primary or secondary. In a recent study, Frøkjær *et al* found hyperreactivity and impaired coordination to bag distension of the esophagus in IDDM patients, which indicates that a neuronal dysfunction is responsible for the hyperreactivity[19]. This dysfunction can theoretically be addressed to the mechano-sensitive afferent fibers, inter-neurons or efferent fibers located in the esophageal wall. However, impaired extra-intestinal pathways can potentially also affect the motor response. In another study, Frøkjær *et al* demonstrated that IDDM patients had decreased sensitivity to the stimulations of the esophagus and duodenum[41]. This was accompanied by an increase in somatic referred pain areas to the gut stimulation (Figure 1). As the referred pain is a result of convergence between visceral and somatic afferents on central neurons, the findings are indirect evidence for central hyperexcitability and neuroplastic changes. Altogether, this indicate that the neuronal changes can be located both peripherally (receptors, nerve fibers, ganglions) and in the CNS. Sensory nerve dysfunction in the gut may explain why diabetic patients with severe GI motility disorders often do not suffer from the GI symptoms expected from the abnormal motility and motor function. On the another hand a large subgroup of patients with longstanding DM suffers from severe GI symptoms[2,26,27]. This overall hyperalgesia/hypersensitivity is in contrast to the peripheral autonomic afferent neuropathy that most likely impairs the perception from the GI tract. The mismatch between hypo-and hypersensitivity can likely be explained by an impaired balance between peripheral and central neuronal changes. Hence, the impaired function of the peripheral afferent nerves is likely counterbalanced (or even overruled) by increased central (spinal and/or brain) neuronal excitability and this balance may determine the symptoms in individual patients.

It is likely that different pathophysiological mechanisms contribute to the peripheral and central neuronal changes in DM, including metabolic alterations, microvascular changes, and inflammatory changes[132]. Hypoxia, hyperglycemia, and increased oxidative stress in DM contribute directly and indirectly to Schwann cell dysfunction[133]. This will result in impaired paranodal barrier function, damaged myelin, reduced antioxidative capacity, and decreased neurotrophic support for axons. Furthermore, the direct effects of prolonged hyperglycemia through the glycation on nervous tissue are also important in the development of DAN[134-136].

Morphological and biomechanical remodeling

Although the hyperglycemia and neuropathy seem to be the main mechanisms to the motor-sensory dysfunction in the upper GI tract in DM, the question remains whether the disordered motor and sensory functions of the GI tract are only due to the neuronal changes and dysfunction or if primary diabetes-induced structural and biomechanical changes in the GI tract also play a role? Data on the biomechanical properties are crucial for the understanding of the motor function of the GI tract because, (1) peristaltic motion that propels the food through the GI tract is a result of interaction of the passive and active tissue forces and the hydrodynamic forces in the food bolus and (2) remodeling of the mechanical properties reflects the changes in the tissue structure that determine a specific motor dysfunction. Therefore, the morphological and biomechanical remodeling of the GI wall may also be an important factor in the pathogenesis to the GI motor-sensory dysfunction in the diabetic patients.

Esophageal remodeling: Many studies have shown that DM causes morphological changes and biomechanical remodeling in the esophagus. Yang *et al*[13] in the *in vitro* study on the STZ-induced diabetic rat esophagus found that the wall thickness and cross-sectional wall area increased after the induction of diabetes. Twenty-eight days after the diabetic esophagus became stiffer both in shear and in the longitudinal direction. The esophageal remodeling was also found in NIDDM rat esophagus[18]. In the diabetic rats the opening angle and residual strain distribution in the outer surface of the wall decreased, the collagen fraction in the mucosa-submucosa layer and the passive circumferential stiffness of the esophageal wall increased (Figure 2). Furthermore, the esophageal weight per length, wall thickness and cross-sectional wall area increased in the NIDDM rats.

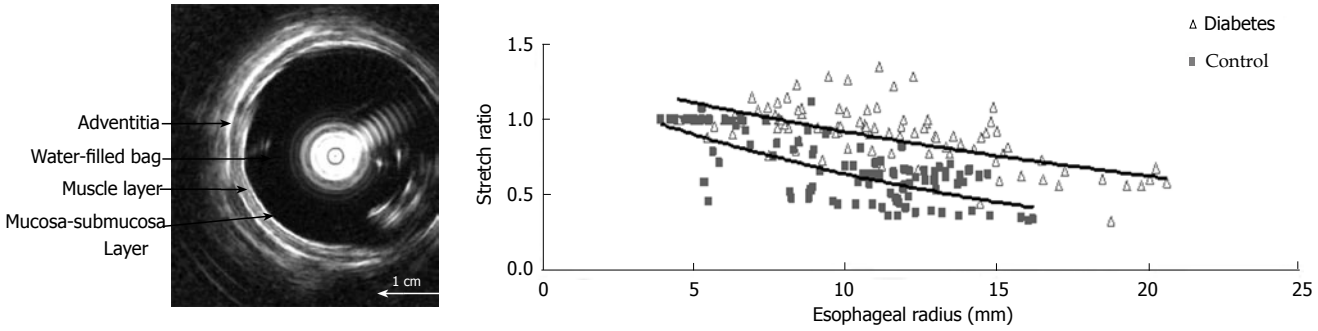

Figure 3 Left: Cross-sectional ultrasound image of the distended distal esophagus allows identification of the esophageal layers, i.e. mucosa-submucosa, muscle and adventitia layers. The white round shadow in the center is caused by the intraluminal ultrasound probe. Right: The distension-induced change in longitudinal stretch ratio is illustrated as function of the esophageal radius. The curves were obtained during smooth muscle relaxation with butylscopolamine. Exponential trend lines of the patients and controls are shown. The shortening during distension was clearly reduced in the diabetic patients.

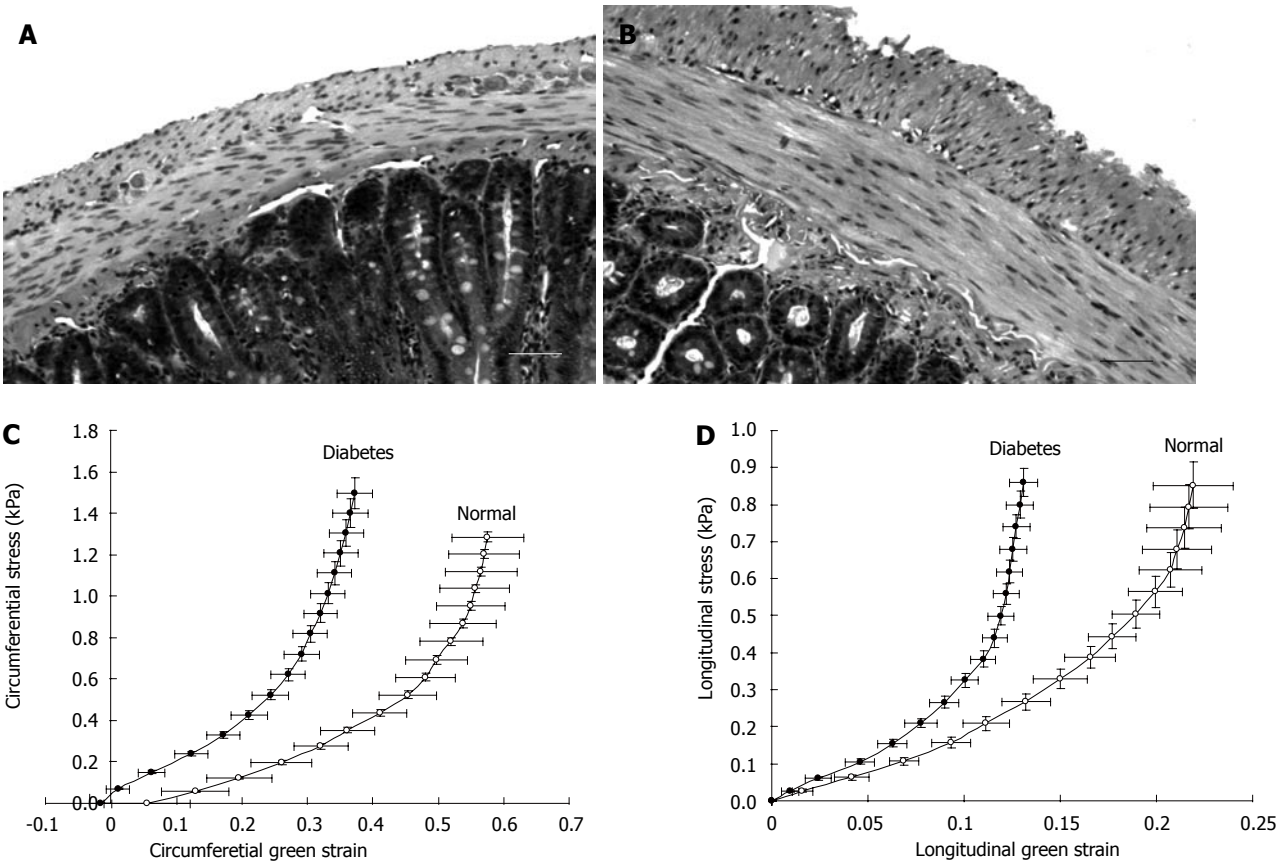

Figure 4 The micro-photographs show the normal (**A**) and 4 wk STZ-induced diabetic (**B**) duodenal histological sections. It clearly demonstrates that the muscle and submucosa layers in the diabetic duodenum became much thicker than in the normal duodenum. The bar is 100 μm. Correspondingly, the circumferential (**C**) and longitudinal (**D**) stress-strain relation curves of the duodenum in the STZ-induced diabetic rats shifted to the left indicating the wall became stiffer.

One previous human study reported that among six cases of diffuse muscular hypertrophy of the esophagus, four cases were associated with DM[137]. Recently, Frøkjær *et al*[19] in an human study found an increase in esophageal wall thickness and altered deformation to the distension with reduced longitudinal shortening and the radial stretch in IDDM patients (Figure 3).

Stomach remodeling: A morphological study in DM rats demonstrated that the gastric mucosa thickness increased in DM rats compared with controls[138]. Remodeling of the interstitial cells network of Cajal in the stomach were found both in animals and humans with DM[139-141]. A histopathological study of the human stomach in DM patients with severe gastroparesis showed prominent collagenization and smooth muscle atrophy of the muscle layer[142]. Regarding the biomechanical remodeling of the stomach only one report by Liao *et al* is available[17]. The rat stomach was distended *in vitro*. Gastric compliance, the surface tension, and circumferential and longitudinal deformation-pressure curves were calculated based on three-dimensional ultrasound reconstructions of non-diabetic and diabetic stomach models. In experimental DM, gastric compliance was lowered both in the non-glandular stomach (proximal part) and the whole stomach (Figure 4). Fur-

Table 3 Morphological and biomechanical changes of small intestine in DM

Morphological change	Biomechanical change
Intestinal weight, length, and weight per unit length↑	Opening angle and residual strain in duodenum↓
Surface area of mucosa↑	Opening angle and residual strain in jejunum and ileum↑
Number of goblet cells per villus↑	Circumferential stiffness of the intestinal wall↑
Smooth muscle mass↑	Longitudinal stiffness of the intestinal wall↑
Different layer thickness↑	Stress relaxation of small intestine↓
Proliferating cell nuclear antigen (PCNA) ↑	
interstitial cells of Cajal volume↓	

thermore, the circumferential stiffness in the non-glandular part increased. The structural changes of the stomach due to DM may together with the sensory and motor nerve dysfunction contribute to the delayed gastric emptying and the symptoms in diabetic patients.

Small intestinal remodeling: Many human[2,11,143,144] and animal [2,3,10,145-148] studies have shown that DM causes morphological alterations in the small intestine (Table 3). However, only few data exist relating to biomechanical remodeling of the small intestine in DM[12]. Recently we did a series of studies on the morphological and biomechanical remodeling of small intestine in the STZ-induced diabetic rats[14-16]. The major findings in our studies are summarized in Table 3 and briefly showed that the stiffness of the diabetic intestinal wall increased in a time-dependent manner (Figure 5). The viscoelastic behavior of intestinal wall also changed during the development of diabetes (Table 3). The stress-strain distribution and viscoelastic behavior mainly reflects the elastic properties of intestine. The changes in elastic properties reflect the structural remodeling of the intestinal wall during the diabetic development.

Advanced glycation end products (AGEs) produced during the development of DM likely relate to the morphological changes and biomechanical remodeling of the GI tract in DM. AGEs are generated by the sequential non-enzymatic glycation of protein amino groups and by oxidation reaction[149]. The accumulation of AGEs in tissues will alter the structure and function of matrix proteins[150]. Studies on DM and ageing show that AGEs are causing cross-linking of collagen molecules responsible for basement membrane thickening and loss of matrix elasticity[151-153]. AGEs may contribute to the diabetic GI morphological and biomechanical remodeling by at least two major mechanisms. The first is receptor-independent alteration of the extracellular matrix architecture by non-enzymatic glycation and the formation of protein cross-links. The second mechanism is receptor-dependent and consists of modulation of cellular functions through ligation of specific cell surface receptors[154-156]. Sanchez SS and coworkers have demonstrated that the expression of small intestinal extracellular matrix proteins have changed in STZ-induced diabetic rats[152]. Therefore, the nonenzymatic glycation of the GI tissue induced by long-term hyperglycemia seems to be an important mechanism behind the GI wall remodeling in DM.

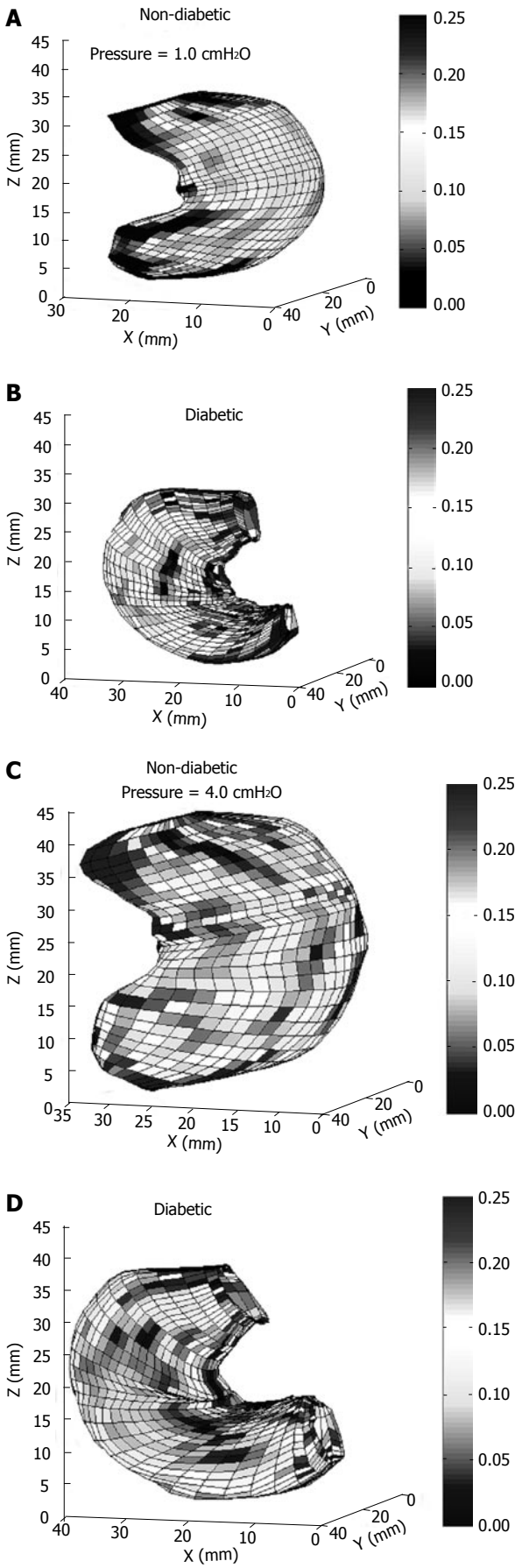

Figure 5 The circumferential curvature at the distension pressure of 1.0 (**A**,**B**) and 4.0 (**C**,**D**) cmH2O in the non-diabetic (**A**,**C**) and the diabetic stomach (**B**,**D**). The circumferential deformation of the stomach in the non-diabetic rats was significantly bigger than that in the diabetic Goto-Kakizaki (GK) rats, indicating the stiffness of the diabetic gastric wall increased in the diabetic GK rats.

Implication of GI remodeling on mechanosensory assessments: Mechanosensation is of fundamental importance for the GI function. The mechanosensitive nerve endings exist extensively in the GI tract where they serve a critical role in homeostasis. Several mechanoreceptor-like structures have been identified such as intraganglionic laminar nerve endings (IGLE) and intramuscular arrays (IMA) in the GI tract[157-164]. The mechanosensitive afferents in the intrinsic and extrinsic pathways were described as low-, wide-dynamic- or high-threshold tension-receptors[165]. Therefore, the GI tract structure as well as the tension, stress and strain distribution in the wall is important for the GI sensory and motor function. The GI wall structure or deformation changes in the DM will alter the relative positions of the mechanosensitive afferents (zero setting of the mechanosensitive afferents)[166]. The biomechanical remodeling in the DM such as alterations of residual strain and stress distribution and increase the wall stiffness will alter the tension and stress distribution of the mechanosensitive afferents. As results, the perception and motility of the GI tract will change as well. Hence, the morphological changes and biomechanical remodeling of GI tract in the DM is likely to affect the function of mechanosensitive afferents in the GI wall and further affect the motor and sensory function. However, so far the data are sparse about the association between the morphological and biomechanical remodeling of GI tract and the motor-sensory dysfunction in the DM[19,41]. The multimodal stimulations have proven accurate and reliable in the assessment of visceral sensation in several studies[167-169]. The geometry data of GI tract can be obtained by impedance and cross-sectional imaging such as ultrasound[170-172]. Combined with pressure recordings, biomechanical parameters such as tension, stress and strain can be obtained and correlated to the symptoms and mechanosensory data in the DM subjects[41]. Therefore, combined studies the GI motor-sensory dysfunction and morphological and biomechanical remodeling in the diabetic GI tract will improve our understanding about the pathophysiology of GI disorders in the DM patients. In the future, animal studies are needed to investigate the passive and active biomechanical and mechanosensory properties of the GI tract related to DM. The experiments should simulate the physiological conditions both *in vivo* and *in vitro* in normal and diabetic animals. The identification of mechanosensitive afferents and enteric nerve responses to the different stimuli will obviously be beneficial to understand the mechanisms of GI motor-sensory dysfunction in the DM.

In conclusion, GI symptoms are frequent in the diabetic patients and are associated with sensory-motor abnormalities, such as impaired perception and motility of the GI tract. The pathogenesis of abnormal GI sensory-motor function in DM is clearly multi-factorial. DAN seems to be the major factor in the pathogenesis of disordered GI motor and sensory functions in DM. Hyperglycemia has also been shown to impair GI motor and sensory function. Furthermore, the morphological changes and biomechanical remodeling of the GI wall may compromise the GI motor function and affect the function of the mechanosensitive afferents in the GI wall. Studies of the relation between the GI motor-sensory dysfunction, morphological changes and biomechanical remodeling in the diabetic GI tract may shed of more light to understand the mechanism of GI motor-sensory dysfunction in the diabetic patients. This knowledge may prove to be valuable in the development of new treatment strategies, which can also be evaluated with the developed methods. New treatments that may be based on the present knowledge and methods are: (1) neurostimulation of afferent visceral nerves, i.e. gastric electric stimulation, (2) agents which can break down already formed glycation end product protein-protein cross-links, and (3) modulation of the central nervous system excitability by drugs.

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TOPIC HIGHLIGHT

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Morphology and motor function of the gastrointestinal tract examined with endosonography

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Abstract

Endosonography is a useful tool for studying the morphology and motor function of the gastrointestinal tract. Intraluminal ultrasonography is the common denomination of ultrasound examinations using intracorporal transducers which are inserted into the GI tract. Thus, the visceral wall and adjacent structures can be imaged in detail. This review describes the usefulness of endosonography in gastroenterology, in particular with respect to studies of the biomechanical and motor function of the gastrointestinal tract. New techniques such as 3-D EUS, elastography and strain rate imaging are discussed.

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Key words: Intraluminal ultrasonography; Gastrointestinal tract; Imaging; Biomechanics

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INTRODUCTION

Disorders of the gastrointestinal (GI) tract may be examined using endoscopy, X-ray, radionuclide studies, electromyography, magnetic resonance imaging (MRI) and manometry. Specific analysis of structural changes occurring within the GI wall is, however, difficult to study by these techniques. Intraluminal ultrasonography (endosonography (ES), endoscopic ultrasonography (EUS) is the common denomination of ultrasound examinations using intracorporal transducers which are inserted into the GI tract. Thus, the visceral wall and adjacent structures can be imaged in detail[1-3]. Acoustic coupling is usually achieved by filling water into the lumen or into a balloon around the ultrasound transducer. Alternatively, the ultrasound probe can be applied directly on the endoluminal surface.

Endosonography is currently mostly performed in the upper GI tract and rectum. However, endosonographic imaging of the colon and distal ileum as well as intraductal ultrasonography (IDUS) of biliary and pancreatic ducts are frequently performed with miniprobes. Dedicated echocolonoscopes are also available, but these have so far gained limited clinical application.

ENDOSONOGRAPHIC INSTRUMENTS

Echoendoscopes combine endoscopy with integrated ultrasound transducers. These instruments can be classified both according to the type of endoscope and the US system. Echoendoscopes may have radial or curvilinear array US transducers using frequencies between 7.5 and 30 MHz. These systems are being continuously improved. The possibility to combine echoendoscopes with conventional ultrasound machines has allowed the use of different ultrasound modalities like B-mode, M-mode and Doppler technology. Ultrasound elastography is a new method with the potential of separating normal from pathological tissue. Recently, elastography and contrast enhanced ultrasound have been applied also in endosonographic imaging[4]. US miniprobes have a diameter which allows the probe to be inserted through the biopsy channel of a conventional endoscope or a suitable catheter (Figure 1). Currently, available US miniprobes comprise mechanical sector, electronic phased arrays, or single crystal linear compound systems operating with frequencies between 12 and 30 MHz. Systems that combine linear compound scanning with mechanical sector scanning or M-mode registration are also available making it possible to switch between these modalities.

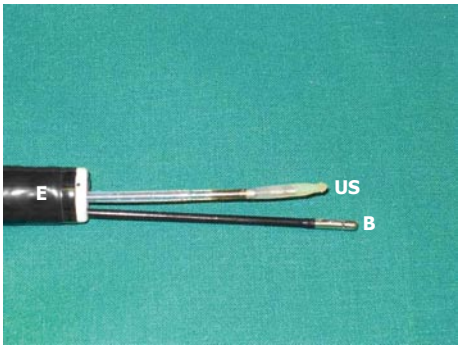

Figure 1 An endoscope with two channels. A miniature ultrasound transducer (US) and a biopsy forceps (B) are inserted through the channels.

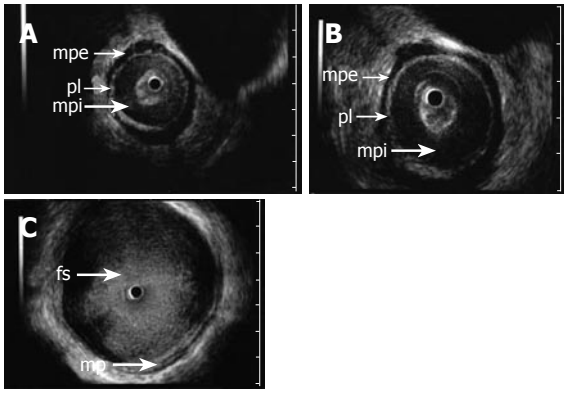

Figure 2 ES images (**A** and **B**) obtained with a 15 MHz miniprobe in a patient with achalasia showing the longitudinal, outer layer of the proper muscle (mpe) separated from a thickened circular, inner layer (mpi) by an echogenic layer corresponding to the localization of the plexus myentericus (pl). **C**: shows a dilated esophageal lumen with food content (fs). A normal aspect of the proper muscle is seen.

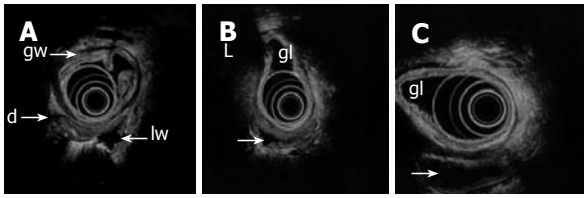

Figure 3 Endosonography with an echoendoscope (12 MHz frequency is used) placed in the gastric lumen (gl) showing the layers of the gastric wall (gw) and GI loops outside the stomach. Lumen is partly collapsed (d), partly waterfilled (arrows). Motility and contractions of native GI loops may be studied. High frequency miniprobes are less suitable for this purpose due to higher frequencies and small transducer diameter.

The small size and high frequencies of miniature probes reduce the size of the US image and the penetration depth of the US beam into the tissue as compared to echoendoscopes. However, small diameter probes can be passed into stenotic areas not traversable with conventional echoendoscopes. Doppler sonography may provide important information about vascular flow and separate vessels from other echo-poor structures. Some echoendoscopes have integrated Doppler technology and Doppler miniprobes for transendoscopic use have also been developed[5]. Other ultrasound systems designed for use within the GI tract do not have an optical system. Transrectal probes have been used for imaging the prostate, bladder, uterus, and rectum. These probes are either linear array or mechanical sector.

ENDOSONOGRAPHIC ASSESSMENT OF GI MOTILITY AND BIOMECHANICS

In recent years ultrasound has been applied in studies of the biomechanical function of the GI tract. In this sense biomechanics should be understood in a much broader sense than the term motility. Motility considers active properties such as peristaltic contractions whereas biomechanics deals with active and passive forces and deformation of the tissue. Biomechanical alterations and abnormal motility in the GI tract is found in several diseases which may be localized in the organ itself or related to the nearest surroundings or to systemic diseases. Luminal manometry has so far been the most widely used method to study GI tract physiology. However, despite the development of high-resolution manometry systems there are still problems in the investigation of GI motor function. The strength of a contraction may vary considerably, thus giving more or less reliable manometric registrations. Furthermore, respiration, aortic pulsation and cardiac contractions may affect the recordings.

The presence of probes in the GI lumen has an influence on physiology and biomechanical studies should therefore ideally be examined without intraluminal devices. ES miniprobes combine the advantages of high ultrasound frequencies with small transducers, allowing detailed images of GI wall layers to be obtained with less influence on the GI lumen than the larger echoendoscopes. Hence, these probes have been regarded suitable for motility studies of the GI tract, especially when applied in lumina with small diameter, like the esophagus (Figure 2). However, echoendoscopes can also be used for examination of motility if positioned in wider lumina like the stomach. Since echoendoscopes generally utilize lower ultrasound frequencies and larger ultrasound probes they can also be used to study motility of neighbouring GI structures (Figure 3). Endosonographic real time B-mode imaging alone or in combination with other methods, as manometry and electromyography, have been used for studying correlation between the pressure and electric potential changes and structural changes in the GI wall. Correct interpretation of ultrasound images of the GI wall is crucial when applying this technique for detailed examination of physiological characteristics. The GI wall consists of mucosa, submucosa, muscularis propria and adventitia/serosa. Muscularis propria has an inner circular and an outer longitudinal layer that is separated by the myenteric plexus.

The normal GI wall is imaged by ultrasonography as a layered structure consisting of 3-9 layers depending on the thickness and structure of different layers, ultrasound frequency, and applied pressure on the wall[6,7]. In most cases, a five-layer structure of the wall can be demonstrated, as determined by the histological layers and the appearance of interface echoes (Figure 2, Figure 4). When the visceral wall is imaged with an endoluminal transducer, the interface between the luminal contents and

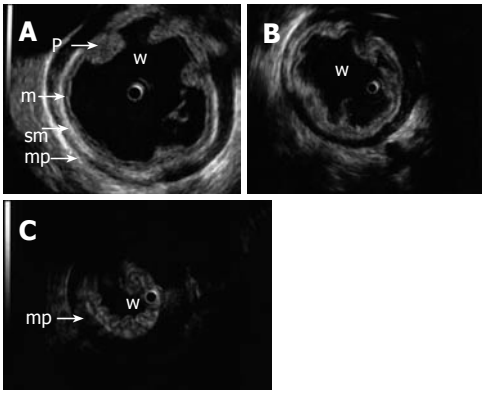

Figure 4 Contraction sequence (**A**, **B**, **C**) demonstrated with a 15 MHz miniprobe in a water-filled (w) stomach. Gastric fold (p), mucosa (m), submucosa (sm), proper muscle (mp).

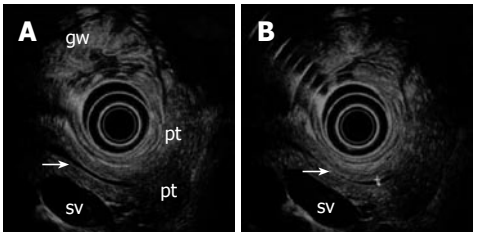

Figure 5 A normal pancreas imaged from the stomach with a 6 MHz echoendoscope. The compressed posterior gastric wall is closely related to the surface of the pancreas. The pancreatic duct is seen (arrow) with minor (**A**) and slightly increased transducer pressure (**B**). The non-compressed gastric wall with folds (gw) is seen on the opposite side of the transducer. Pancreatic tail (pt), splenic vein (sv).

the mucosa accounts for the first, echo-rich layer. The rest of mucosa appears as a second, echo-poor layer. The third, echo-rich layer usually includes the interface between the lamina propria and muscularis mucosae, the submucosa, and the interface at the border of the muscularis propria. In a five-layer structure, the rest of muscularis propria is imaged as an echo-poor fourth layer. The fifth, echogenic layer represents the outer wall delineation and is an interface echo at the serosa or adventitia level.

Sometimes, the use of high-frequency ultrasound produces an interface echo between the different aspects of the muscularis propria, probably reflecting the connective tissue embedding the myenteric plexus. A thick muscularis mucosa may also be identified as an echo-poor layer dividing the third, echogenic layer. Hence, the ultrasonographic wall structure may consist of seven or even nine different layers[6,8].

CLINICAL APPLICATIONS OF ENDOSONOGRAPHY IN BIOMECHANICS AND MOTILITY

Esophagus is often associated with motility disorders which can be studied with different techniques. Endosonography has been regarded as a suitable tool for examination of esophagus because this organ is a straight tube with a small diameter. McCray *et al*[9] studied the high pressure zone between the esophagus and stomach (lower esophageal sphincter and extrinsic crural diaphragm) in normal subjects with a dual high resolution endosonography-manometry catheter. This catheter recorded simultaneously manometric pressures and corresponding ultrasound images while being pulled through the distal esophagus. They found that simultaneous high-resolution endoluminal sonography and pressure manometry separate the two components of the high-pressure zone in the distal esophagus.

Endoscopic ultrasound findings have also suggested that an association exists between sustained longitudinal layer contraction in the esophageal body and chest pain of unknown origin[10]. Furthermore, Nicosia *et al*[11] combined manometry and ultrasound and used conservation of mass principles to quantify local axial shortening of the esophagus. A relationship between contractions in the circular and longitudinal muscle layers during swallowing was established. They also made 3D (space-time) reconstructions of the inner wall of the circular muscle layer for representative swallows. Takeda *et al*[12] used combined ultrasound and manometry to determine circumferential stress-strain properties in the esophagus. In a study by Taniguchi *et al*[13] the sheep esophagus was investigated with M-mode echoesophagram recording and manometry with the goal to measure simultaneously changes in the thickness of the individual esophageal wall layers and the corresponding changes in intraluminal pressure. The maximum manometric pressure, the esophageal wall layer thickness, and the duration of contraction were measured. Swallowing events up to one hour were recorded and in one trial both dry and wet swallows were compared. All swallowing events produced simultaneous increase in intraluminal pressure and esophageal wall thickness. The thickness of the inner (circular) muscle layer increased in dry as well as wet swallows.

The combination of manometry with a 20 MHz ultrasound transducer has also been used to correlate wall thickness, luminal diameter and contractive activity in healthy volunteers[14]. The increase in muscle width by ultrasonography was found to correlate with the increase in luminal pressure by manometry. Recently, Hoff *et al*[15] described a multimodal device inserted into esophagus combining bag distension, manometry, high frequency intraluminal ultrasound, laser Doppler flowmetry and symptom registration. Preliminary experience indicates that these modalities can be used in combination without significant technical influence on each other. Thus, this method can provide a basis for further studies, probably within both the upper and lower GI tract.

Achalasia

Achalasia is a disorder in esophageal motility and is defined by its typical pattern at manometry. The diagnosis can be supported by findings at barium radiography that may show delayed passage through the lower esophageal sphincter and dilatation of the body of the esophagus. At manometry, achalasia is characterized primarily by aperistalsis of the esophageal body and incomplete relaxation of the lower esophageal sphincter. In autopsy

studies of patients with achalasia, the thickness of the lower esophageal sphincter is increased. Using high frequency US probes, it has been shown that the thickening of muscularis propria mainly appear in the inner circular portion (Figure 2). Thickening of the circular layer of the muscularis propria at the cardia may support the diagnosis of evolving achalasia[16-23].

A study of the esophageal wall in patients with newly diagnosed achalasia has also been performed using a sector scanning echoendoscope with 12 MHz transducer frequency. The endosonographic findings were correlated with manometric features and clinical symptoms. In these patients, an increase in total wall thickness and thickness of the muscularis propria was found at the level of the lower esophageal sphincter and the proximal 5 cm of the sphincter[16]. Miller *et al*[21] used a 20 MHz radial ultrasound transducer to examine the width of the total muscularis propria, and the circular and longitudinal smooth muscles at the lower esophageal sphincter in 29 patients with achalasia, comparing them with 19 normal subjects. They found that the mean width of these muscles was increased in patients with achalasia. However, because of overlap in muscle width thicknesses between these groups it was concluded that a thickened muscularis propria cannot be used to differentiate clinically between patients with achalasia and normal controls. Nevertheless, they found that the mean diameter of both the longitudinal and the circular smooth muscle layers at the lower esophageal sphincter were wider in patients with achalasia than in normal subjects. Trowers *et al*[22] used a single crystal transendoscopic probe providing linear images. Six achalasia patients were examined, demonstrating the thickness of the muscularis propria to be increased four-fold, and all patients were abnormal compared to controls.

Scleroderma

Patients suffering from systemic sclerosis have frequently clinical gastrointestinal manifestations, especially excessive deposition of collagen in the esophagus which may cause swallowing problems. Endoluminal US seems capable of defining morphologic as well as physiologic abnormalities of the esophagus in patients with scleroderma of the esophagus. Lieu *et al*[24] examined four patients and found that US images correlated with established pathologic descriptions like smooth muscle atrophy, variable fibrosis, and collagen deposition. Increased echogenicity and thinning of the muscularis propria, consistent with fibrosis and atrophy of the smooth muscle were observed. Miller *et al*[25] used a 20 MHz ultrasound transducer in examining 13 patients suffering from systemic sclerosis and 13 healthy subjects. Also postmortem autopsy study was performed to compare histopathological abnormalities with sonographic findings in the esophagus. In the postmortem ultrasound study of two cases, hyperechoic echoes were seen in the normally hypoechoic muscularis propria. These findings corresponded to fibrosis found in histological examinations. Hyperechoic abnormalities were ultrasonographically seen in the muscularis propria in patients but not in normal controls. A strong positive correlation between esophageal hyperechoic and manometric abnormalities was also found.

Nutcracker esophagus

Patients with nutcracker esophagus (NE) suffer dysphagia and/or chest pain due to strong peristaltic contractions. High-pressure amplitudes are found at manometry. Melzer *et al*[26,27] used an echoendoscope to examine the thickness of the muscularis propria at the gastroesophageal junction and in the lower, middle, and upper esophagus in patients with nutcracker esophagus. The muscularis propria was thickened in one-third of the patients. This thickening, however, did not correspond to the location and magnitude of the manometric abnormality and did not correlate with the clinical presentation. They also reported a patient with NE and esophageal pressures exceeding 800 mmHg in whom endoscopic ultrasonography demonstrated a markedly thickened esophageal muscularis propria. The hypertrophy of the muscularis propria extended from the gastroesophageal junction upwards to the middle esophagus.

Reflux esophagitis

Esophageal dysfunction can be seen in patients with reflux esophagitis. Important pathophysiological factors are impaired esophageal acid clearance and a hypotonic lower esophageal sphincter. The intraluminal esophageal pressure is related to the contraction of the inner circular smooth muscle. Dysfunction of the lower esophageal sphincter area may cause esophageal reflux disease. Contraction of the longitudinal smooth muscle during swallowing causes axial shortening of the esophagus. This muscle may also stiffen the esophagus and thus generate intraluminal pressure during peristalsis. In patients with reflux esophagitis, inflammatory changes are present, affecting all or individual wall layers in the distal part of the esophagus. Periesophageal inflammation may also be present and, depending on the degree of total inflammation, it may affect the esophageal motility and thus also acid clearance. These changes can usually be imaged by ultrasound miniprobes, which thus may provide insights into the pathophysiology of reflux esophagitis.

GASTRODUODENAL MOTILITY

Endosonography of the stomach and duodenum is in essence performed in the same way as in the esophagus. However, endosonography can be technically more challenging in wide and curved organs. Transabdominal ultrasound is applied in studying gastroduodenal motility, also in combination with intraluminal pressure devices. Recently, mechanosensory properties in the human gastric antrum were evaluated using transabdominal ultrasound scanning during volume-controlled antral distension[28,29]. Furthermore, a device which combines endosonographic real - time imaging with intraluminal volume-controlled bag distensions has been developed also for use in large lumina. Thus, detailed images of GI wall layers corresponding to the area of bag pressure can be obtained. Arslan *et al*[30] investigated duodenum with high-frequency ultrasound to study wall layers and motility during allergenic

provocation. A 20 MHz miniature ultrasound probe was inserted through a nasoduodenal tube and ultrasound examination was performed before and after provocation. Clinical symptoms were recorded and the thickness of mucosa, submucosa and muscularis propria was measured directly on ultrasonographic images. Endosonographic changes (i.e. any resolvable wall thickening, contraction or new echogenic layer) were observed in 15 of the 20 patients. Mucosal thickening, changes in the number of wall layers and sustained contractions of duodenum were observed in some patients after provocation. The mucosal thickening could represent mucosal edema in response to provocation. A new echorich layer was observed in two patients, probably caused by an additional interface at the border of a thicker muscularis mucosa due to the allergic reaction.

Rectum, large and small intestine

These organs may be examined in the same way as described for esophagus, stomach and duodenum using dedicated echoendoscopes and rectal probes. However, ES miniprobes, which can easily be inserted through the biopsy channel of standard endoscopes, seem to be a practical method for imaging the colon as well as the rectum. The interpretation of wall layers, organ contractions and other motility aspects is similar to that of the upper GI tract. Endoscopy of the small intestine is now possible in some GI laboratories. New enteroscopes may in the future be integrated with ultrasound transducers making ES/EUS of the small intestine possible. Capsule endoscopy which also is a relatively new method, allows endoscopic imaging of the entire small intestine. If a miniature ultrasound device is integrated in this capsule, EUS of the small intestine may possibly be performed.

NEW TECHNIQUES

Three-dimensional EUS

3D- images are assumed to be easier to understand and communicate than the mental reconstruction of several 2D-images. 3D-techniques are currently under development for most imaging modalities and 3D-EUS may be applied for improved recognition of the GI anatomy and pathological lesions. 3D-EUS images can be obtained using images acquired by echoendoscopes or miniprobes. Mostly, a pullback device has been used to obtain parallel 2D images which are reconstructed to a 3D-image. Acquired ultrasound images can be stored and digitized using frame grabbing but direct digitizing of ultrasound raw data is being increasingly used in new scanners. Advanced software programs containing different rendering or other reconstruction algorithms are used for postprocessing and display of 3D ultrasound data, which can be studied using anyplane slicing, segmentation and volume calculation[31,32].

Elastography

The stiffness of normal and pathological tissue is usually different and may be evaluated by ultrasound which is based on B-mode scanning during compressions (elastography) (Figure 5). Elastography has been applied on the GI tract, kidney, muscle, breast and the heart. The underlying principle is that the deformation of tissue by a mechanical excitation is a function of its mechanical properties. For gastrointestinal purposes, the intraluminal pressure may be used as the excitation force[33,34].

Imaging of ultrasound tissue elasticity has gained considerable interest as a method to differentiate normal from abnormal tissue, particularly malignant from non-malignant disease. However, normal structures also have variable internal sonoelastic properties. When compressed by an ultrasound transducer, the ultrasound image of normal as well as pathological tissue is changed. It has been demonstrated that increased pressure on the GI wall changes the thickness, layers and echogenicity of the wall as imaged by B-mode ultrasonography[7].

Strain rate imaging

Strain rate imaging is a recently introduced technique to measure deformation of biological tissue. The technique is based on tissue velocity imaging, which is an ultrasound technique that provides quantitative information on velocities within a tissue. By color-coded tissue velocity imaging, velocity data from the whole field of view are available simultaneously[35]. This allows extraction of other parameters through spatial and temporal processing of the velocity data. Strain rate and strain are examples of such parameters. The methods have primarily been used in echocardiography, but other uses have also been reported, including measurements of gastric motor function[36].

Further development of specialized EUS instruments can allow the investigators to choose the most appropriate instruments for the separate tasks. Miniature probes, which are less costly and easier to operate, will most likely be available in a majority of endoscopy units within a few years. Different modalities can probably be merged into new multimodal devices making it possible to obtain multiple motility parameters in one procedure. A successful development of such devices can hopefully be achieved on the basis of fruitful collaboration between medicine, technology and soft-ware producers.

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TOPIC HIGHLIGHT

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New perspectives of studying gastrointestinal muscle function

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Abstract

The motor function of the gastrointestinal tract has primarily been studied using manometry and radiography, though more indirect tests have also been applied. Manometry and radiography do not provide detailed information about the muscle properties as can be assessed from studies of muscle properties in muscle strips in vitro. In recent years a technique based on impedance planimetric measurement of pressure-cross-sectional area relations in a distending bag has proven to provide more detailed information about the muscle function in vivo. This review shows examples of new muscle function analysis such as length-tension diagrams, force-velocity curves and preload-afterload diagrams.

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Key words: Muscle analysis; Length-tension; Force; Velocity

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INTRODUCTION

The gastrointestinal (GI) tract is a continuous channel through the body with oral and anal orifices for intake and output. It consists of a series of organs with the biliary tract as a major side-branch. These organs resemble one another in constitution, the elements being variously arranged in such a way as to produce cylinders, spheroids, or intermediate forms[1]. The wall of the GI tract is a laminated composite structure. The general structure is an outer muscle coat consisting of a longitudinal muscle layer and a circular muscle layer. The collagen-rich submucosa layer and a mucosa layers are found inside the outer muscle coat. A third layer of muscle, the muscularis mucosae, exists throughout almost the entire tract. A network of nerves, the myenteric plexus, is embedded in the loose collagen matrix of the intermuscular plane. This set of nerves is essential in the regulation of the contractions of the adjacent musculature, the two layers of the muscularis propria. The motions of the GI tract must, at all times, keep the contents of the tract in their intended place, i.e. to bring them to the site of absorption. This means not only that they accomplish net antegrade flow but also that they mix the contents and move it across the surface where absorption occurs. The contractile patterns and transit varies greatly from one part of the tract to another. The muscle layers provide the forces that distort the cylindrical conduit in the form of contractions. The connective tissue layers provide a framework, or stroma, that determines the passive physical properties of the gut wall.

As stated above, the gross mechanical function of the GI tract depends on the coordinated muscle function and neuromuscular circuits. In bioengineering terms the mechanical properties of the GI tract has mainly been studied in vitro under standardized isometric and isotonic conditions and length-tension and force-velocity characteristics have been described[2]. The muscle function is altered in a number of organic and functional GI diseases, as well as it can be modified by drugs. Despite the importance of the muscle function, only few methods exist to evaluate the mechanics of GI muscle function in vivo. Manometry has become a standard method for clinical motility testing but it merely provides a proxy of the force developed by the muscles and is in many diseases poorly related to the organ dysfunction and symptoms[3].

Heart muscle function has attracted more attention that GI muscle function in the past and pressure-volume loops and ventricular function curves can be generated from measurements obtained *in vivo*[4,5]. Inspired by the cardiovascular research similar "muscle function" curves can now be obtained in the GI tract from impedance planimetric studies and such analyses provide new insight into the behavior and function of the GI tract. The review provides the background for advanced muscle analysis in vivo and takes the readers through some examples.

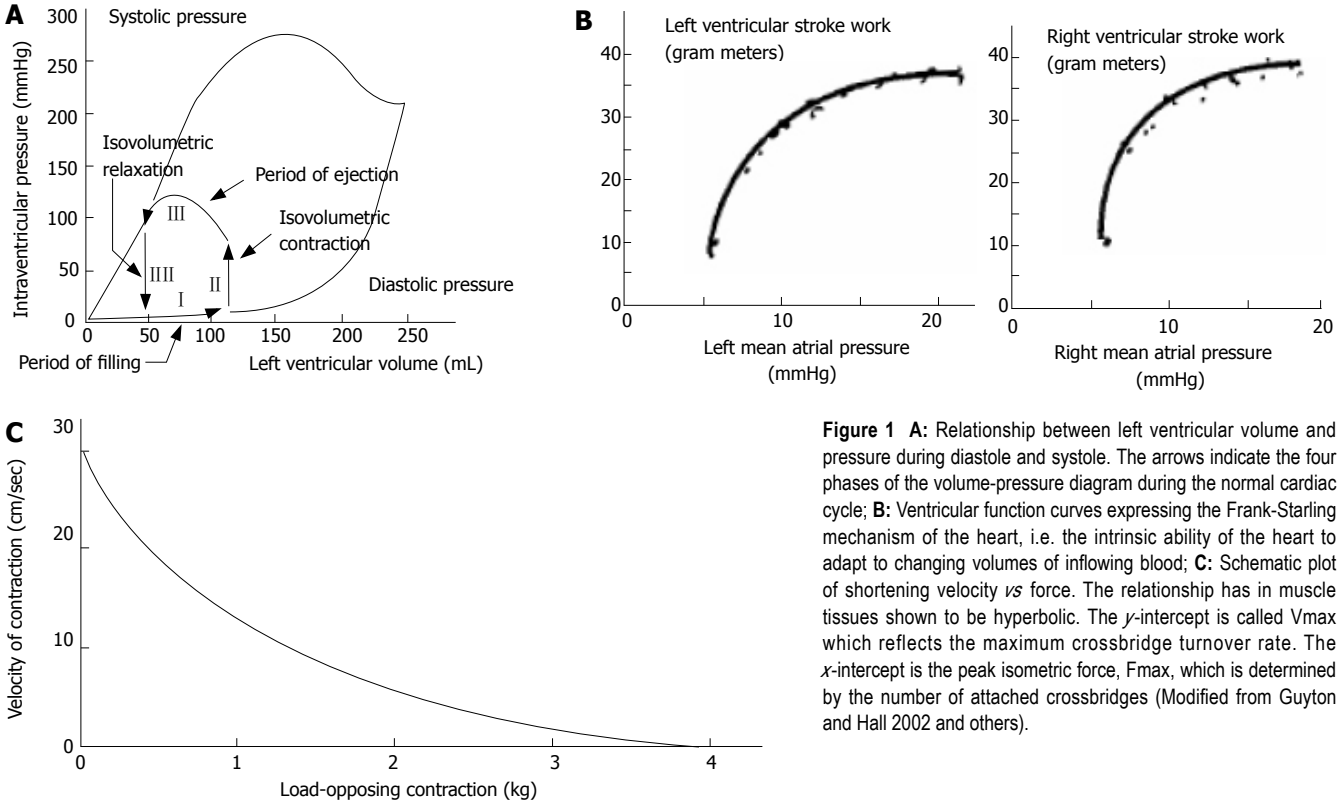

Figure 1 **A:** Relationship between left ventricular volume and pressure during diastole and systole. The arrows indicate the four phases of the volume-pressure diagram during the normal cardiac cycle; **B:** Ventricular function curves expressing the Frank-Starling mechanism of the heart, i.e. the intrinsic ability of the heart to adapt to changing volumes of inflowing blood; **C:** Schematic plot of shortening velocity *vs* force. The relationship has in muscle tissues shown to be hyperbolic. The *y*-intercept is called Vmax which reflects the maximum crossbridge turnover rate. The *x*-intercept is the peak isometric force, Fmax, which is determined by the number of attached crossbridges (Modified from Guyton and Hall 2002 and others).

CURRENT METHODS FOR EVALUATING MUSCLE FUNCTION

The cardiac cycle and cardiac mechanics-function relationship have been studied in much more detail than contractile properties of GI smooth muscle in vivo. Curves are often derived from pressure-volume data where four phases during the cardiac cycle can be identified (Figure 1A), and from stroke work output as function of atrial pressures (Figure 1B). Such data are clinically useful for explaining the pumping mechanics of the left ventricle. One of the best known relationships concerning muscle action is the hyperbolic force-velocity relation[4,6] (Figure 1C). Although this relationship originally was derived for isolated muscle, it has been confirmed for whole striated muscle movements[7]. The curve implies that velocity of muscle contraction is inversely proportional to the load, that a large force cannot be exerted in very rapid movements, that the greatest velocities are attained under conditions of low loading, and that the intermediate values of force and velocity depend on the maximal isometric force.

Most mechanical studies in vitro on GI smooth muscle is isometric length-tension experiments conducted at resting conditions in small organ baths and during stimulation or inhibition with pharmacological substances[2]. Such studies have given valuable information about the regulation of contractions and passive tissue properties.

Only few methods are established for evaluation of GI motor and biomechanical function in vivo. Luminal pressure recordings are the key measurement technique for determining the contractile pattern in many parts of the GI tract. Manometry has been used for more than 50 years to monitor motility in the gastrointestinal tract. These systems often use reliable perfused hydraulic-capillary systems where pressure at a sensor outside the body can be used to accurately measure pressure at a point along a catheter *via* a low flow rate perfused channel. This system has been used extensively to measure force via a pressure measurement in lumens and in liquid filled balloons. Other tests may give a more indirect measure of the motor function. These include transit time studies, pH-metry in the esophagus, radiography, *etc*. The measurement of luminal pressure, however, is not a direct measure of muscle tone and tension. It is a proxy of changes in force generated by the surrounding tissues[8,9].

During recent years, bag distension has become popular as a research tool for GI studies, primarily because mechanical data may be obtained. Furthermore, distension provokes pain and therefore can be used to study the sensory function in patients with functional chest pain[10]. Only few distension studies using inhibitory drugs focused on separating phasic and tonic contractions[11,12]. Recently, tools were developed for studying both the active (phasic and tonic contractions) and passive behavior using impedance planimetric distension before and during administration of inhibitory drugs[9,13,14]. Tension, stress and strain are of principal interest because these parameters give information about the esophageal elastic properties and mechanoreceptors respond more directly to these parameters than to pressure and volume[9]. Furthermore, the muscle function is better evaluated when the forces and tensions can be measured rather than the pressure generated by contractions[1,15]. The most used bag distension technique is the barostat but other techniques such as impedance planimetry and distensions combined

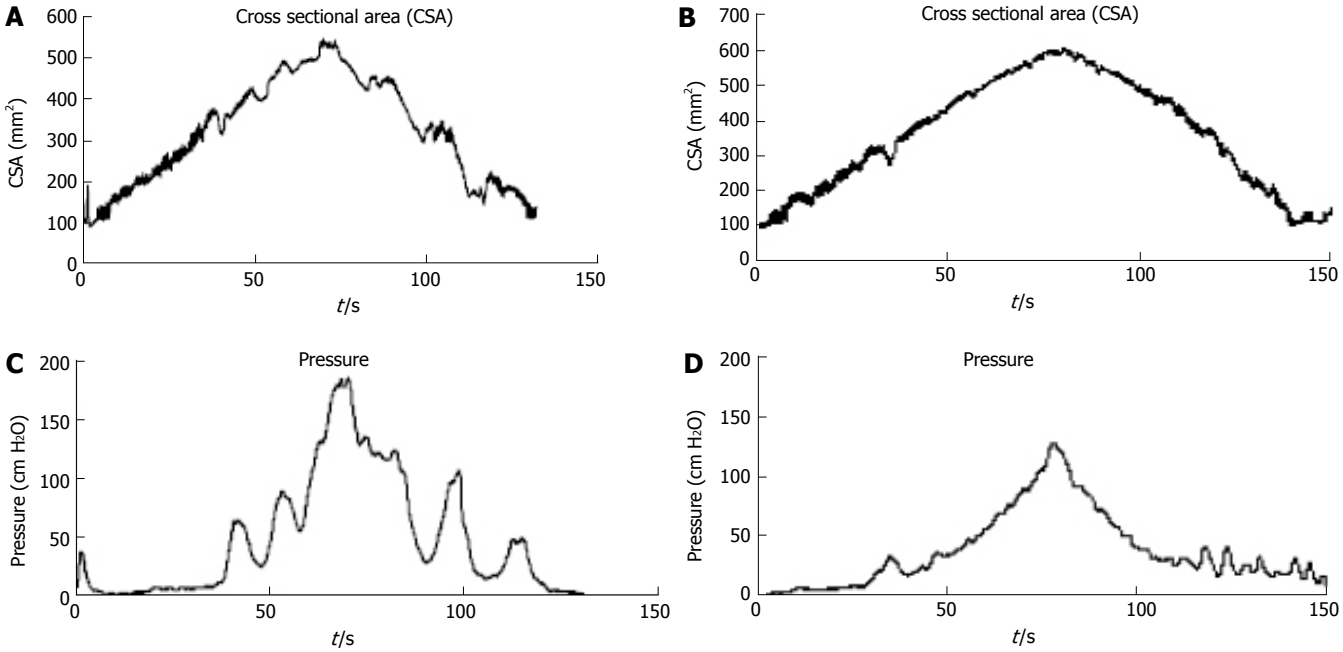

Figure 2 Cross-sectional area (CSA), and pressure as function of time during the distension of esophagus without (**A, C**) and during (**B, D**) the administration of butylscopolamine. Modified from previous publications by Gregersen and coworkers.

with imaging techniques give more detailed geometric information. Rather than analyzing pressure-volume relationships based on the barostat, impedance planimetry enables data on the CSA of the bag during distension to be gathered. CSA shows a better relationship to the diametrical changes in the alimentary lumen than volume[1].

THE IMPEDANCE PLANIMETRIC PRINCIPLE

Impedance planimetry is a technique for performing bag distensions in hollow visceral organs. It has been used to measure CSA together with bag pressure in animal and human studies and the wall tension and strain in the organ can be estimated[1,16]. From Ohm´s law we know that

$$V = IZ$$

where V, I and Z denote the potential difference, the current and the electrical impedance. In the case of a fluid filled bag on a probe with an alternating constant current set up between two excitation electrodes (E), Ohms law will apply for the field between two inner sensing electrodes (S). The impedance depends on the distance (D) between the S electrodes, the conductivity (ς) of the liquid in the bag and the CSA of the bag between the S electrodes. Since

$$Z = \frac{D}{\varsigma CSA}$$

the potential difference can be written as

$$V = \frac{DI}{\varsigma CSA}.$$

If the conductivity of the solution remains constant (the temperature must be constant), the distance between the S electrodes is fixed, and the current source exciting the E electrodes is kept constant, then

$$V = \alpha \frac{1}{CSA},$$

where α is a calibration constant. Thus, the voltage crossing the S electrodes is proportional to the inverse of the CSA. Direct proportionality can be obtained by reciprocation. Data are conditioned and stored on a PC for further analysis.

EXAMPLES OF MUSCLE ANALYSIS

The new analysis consist of *in vivo* (1) phasic and tonic length-tension diagrams; (2) preload-afterload analysis (3) pressure-CSA and tension-radius plots (4) force-velocity and force-power plots.

Basic distention studies

The studies have been done using different step or volume-controlled ramp protocols. An example of CSA and pressure recordings is shown in Figure 2 where a volume-controlled ramp distension was done using an infusion rate of 25 mL/min. The data provide information on the contractile pattern and can be used for computation of elasticity.

Assessment of phasic, tonic and passive length-tension relationship in vivo

Length-tension diagrams can be obtained in vivo in the GI tract using impedance planimetry. Passive curves obtained during administration of butylscopolamine and active curves in terms of both phasic and tonic curves can be generated by analyzing data in accordance with the tracings in Figure 3. For simplicity in the first approach, the circumferential wall tension (T) is calculated according to the law of Laplace for cylindrical structures.

$$T = \Delta P r$$

where T is the circumferential wall tension and r is the bag radius and ΔP is the transmural pressure. The radius is calculated assuming a cylindrical shape of the bag during

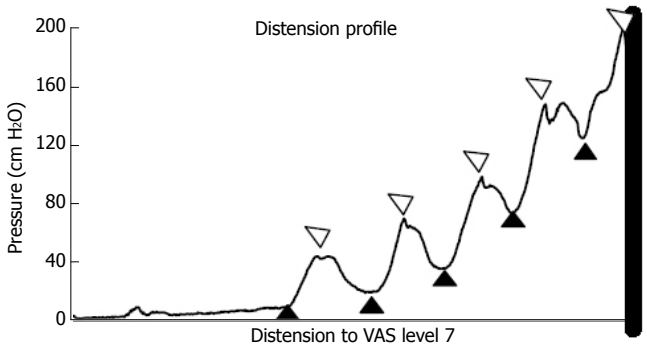

Figure 3 Illustration of a ramp distension curve in the human esophagus. The thick line at the end of the curve is the reverse point. The open symbols above the curve mark the phasic part and the afterload pressure. The closed symbols under the curve mark the tonic part of the distension curve and the preload pressure. The passive pressure is measured from the tonic part of the curve during administration of butylscopolamine. At the symbols pressure and CSA was measured and radius and tension was computed from these values. Modified from previous publications by Gregersen and coworkers.

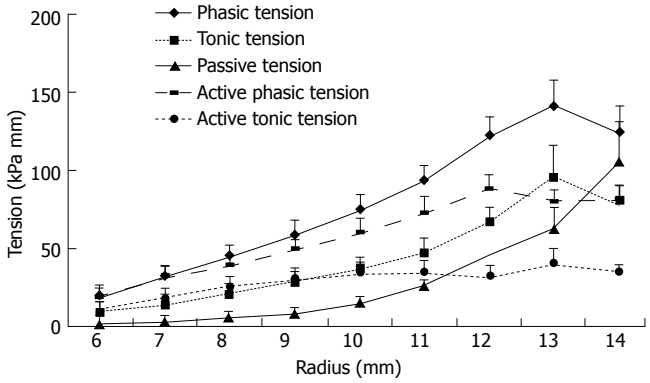

Figure 4 The active and passive tension-radius curves for a subject where the maximum active tension is reached before the moderate pain level (top). The bottom graph shows the averaged data from all volunteers. Mean and SEM values are shown. Modified from previous publications by Gregersen and coworkers.

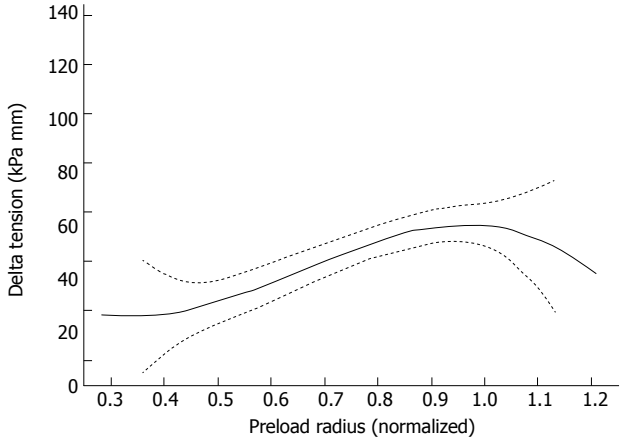

Figure 5 Representation of the change in muscle tension during distension-induced contractions as function of the radius immediately before the contraction (preload-afterload properties) of the esophageal muscles. The solid line is the polynomial fit and the dotted lines represent 95% confidence intervals. Modified from previous publications by Gregersen and coworkers.

distension and by knowing the CSA. The circumferential stretch ratio can be computed as the fractional change in radius (r/r_0) where r is the radius at any distension state and r_0 is the reference radius.

The distentions induce contractile activity. For each contraction, tonic tension (tension computed from pressure and CSA between phasic contractions) and phasic tension (tension during maximum contraction) can be identified from the tension-time curve. Since both active and passive tissue properties contribute to the total tension developed in the small intestine during distension, both the tonic and the phasic tensions must be separated into active and passive components. The passive tension can be derived from the distensions during butylscopolamine infusion and the total tension. Thus, the active tensions can be computed as:

$T_{tonic\text{-}total} = T_{tonic\text{-}active} + T_{passive}$ and $T_{phasic\text{-}total} = T_{phasic\text{-}active} + T_{passive}$

where $T_{tonic\text{-}total}$ $T_{tonic\text{-}active}$, $T_{phasic\text{-}total}$ $T_{phasic\text{-}active}$ and $T_{passive}$ are the total tonic tension, the active tonic tension, the total phasic tension, the active phasic tension, and passive tension, respectively.

The graph in Figure 4 shows averaged data from the normal human esophagus. The passive tension increased exponentially as function of the radius, whereas the tonic and combined phasic-tonic tension increased until a maximum at a radius of 13 mm. This corresponded with the pain threshold. The active tension decreased at higher radii. The major finding was that both active curves exhibited a maximum tension. The active tensions were most important at low distending loads whereas the passive tension was predominant at higher loads. Thus, the esophagus can contribute its largest active force to transport the bolus within the physiological range. The maximum active tension appears close to the pain threshold and before overstretching the esophageal wall. Thus, the esophagus has its physiological function in the ascending leg of the length-tension curve. Exceeding this level it becomes inefficient, like the heart that also functions at the ascending leg of the curve. The maximum active tension is presumably reached at a level of optimum overlap between the sliding filaments in the intestinal muscle cells. When the esophageal wall was stretched to a degree where pain appeared, the passive tension predominated. The passive curves were exponential-like in accordance with findings in other soft tissues. The exponential behavior protects the tissue against overdistension and damage at high luminal pressure loads, while distending easily and facilitating flow in the physiological pressure range.

Preload-afterload diagrams

A way to express the Frank-Starling mechanism in the heart is preload-afterload curve (Figure 1B). In Figure 5 this concept was borrowed from cardiac physiology and it is shown that GI muscle behaves in a similar fashion. The change in tension during individual distension-induced contractions (afterload) was analyzed as function of the radius immediately before the contractions (preload radius). In a similar way the change in radius during distension-induced contractions was plotted as function of the preload radius. These diagrams correspond to heart function curves in terms of the ventricular stroke work as function

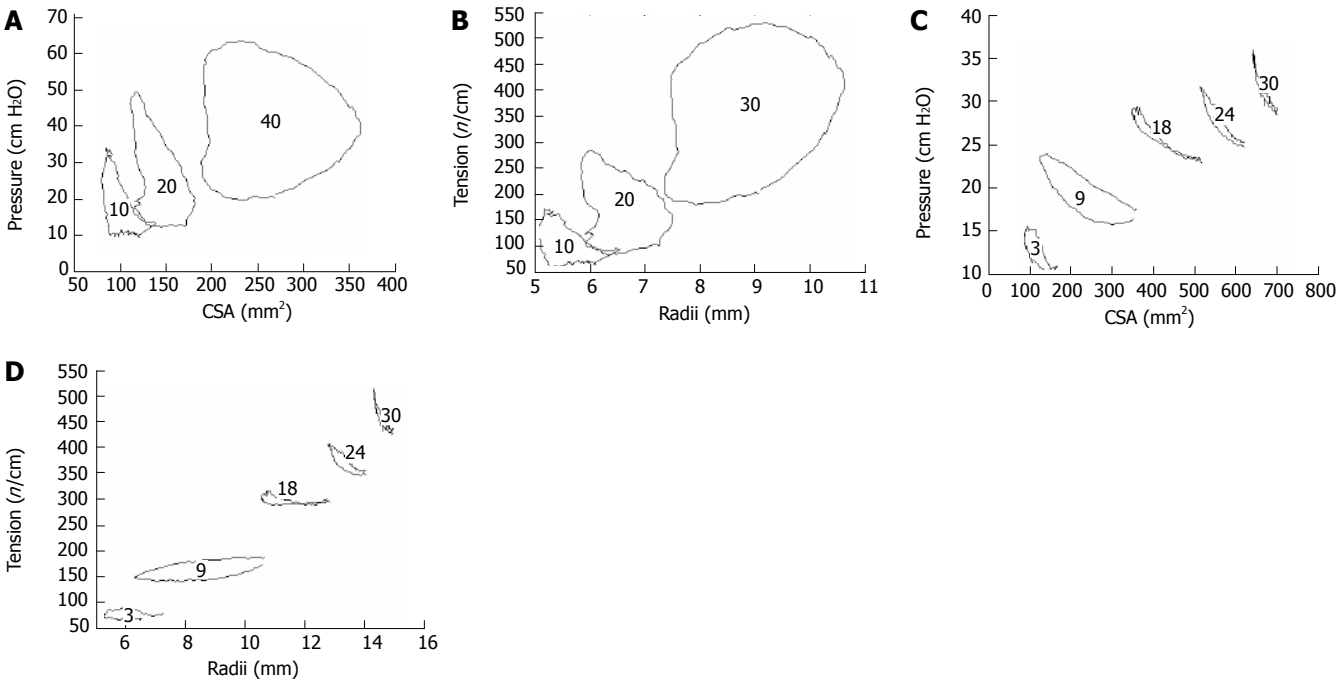

Figure 6 Pressure-CSA and tension-radius relations during esophagus contractions (**A, B**) and during duodenum contractions (**C, D**). The numbers in the figures indicate the volume in ml.

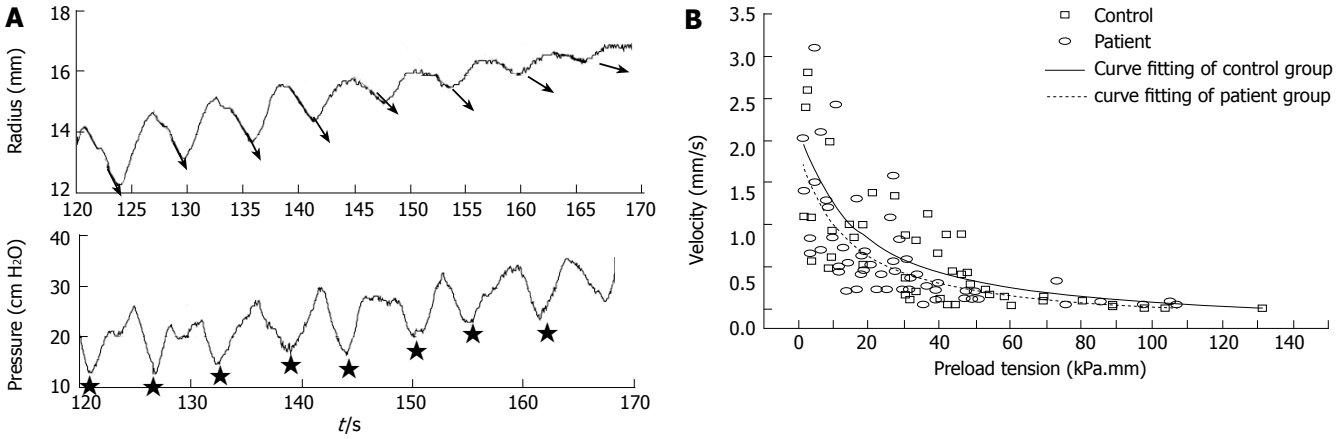

Figure 7 **A:** Arrows indicate that the slope (velocity decreases at high loads. The stars indicate the preload tension; **B:** Force-velocity curves represented as circumferential preload tension-radius shortening velocity and preload tension-circumferential preload tension*CSA rate in SS patients (symbol O and dashed line) and controls (and solid line). The tension-velocity data were curve fitted using Hill's equation. Modified from previous publications by Gregersen and coworkers.

of the mean atrial pressure. Such curves demonstrate the Frank-Starling mechanism of the heart[4,5]. The change in tension during bag-distension-induced contractions increased at normalized radii (radius divided by the radius at the pump reverse point) up to 0.95 (corresponding to radii of 5-12 mm) (Figure 5). This may be called the ascending leg of the curve. At higher radii the change in tension decreased again (the descending leg).

The preload is considered to be the initial muscle length (radius) preceding the contraction during the distension, whereas the afterload is evaluated as the active tension during the contraction. In cardiac physiology the preload is usually considered to be the end-diastolic pressure or radius and the afterload is considered to be the arterial pressure during the systole. The explanation of the Frank-Starling mechanism is that when an extra amount of blood flows into the ventricles, the cardiac muscle itself is stretched to greater length. This causes the muscle to contract with increased force because the actin and myosin filaments are brought to a more nearly optimal degree of interdigitation for force generation. In cardiac physiology the importance of the concept of preload and afterload is that in many abnormal functional states of the heart and circulation, the pressure during filling of the ventricle or the arterial pressure against which the ventricle must contract, or both, are severely altered from the normal. Transferring this concept to GI physiology, the development may have interest for evaluation of normal GI physiology, aging and in the pathophysiology of gastro-esophageal reflux disease, systemic sclerosis, obstruction, achalasia, functional chest

pain, spasms and other esophageal disorders.

P-CSA and tension-radius plots

These curves correspond to the well known pressure-volume curves obtained in the heart (as shown in Figure 1A. The data below was generated from analyzing pressure and CSA data during contractile events at various levels of bag distension in the esophagus and duodenum (Figure 6). It is clearly shown that loops consisting of different "phases" can be determined. However, definition of contraction and relaxation phases seems not as clear cut as in the heart. It is evident that duodenum and esophagus muscle behaves in a very different way. Especially the esophagus curves show a large degree of hysteresis. The area enclosed by the tracing is a measure of the work done during the contraction.

Force-velocity analysis

Figure 1C shows a schematic of the well known force-velocity relationship for muscle tissue and Figure 7 shows data obtained in the GI tract. The tension and shortening velocity were quantified from the contraction waves in the tension-time and radius-time curves simultaneously. For each contraction the preload tension was defined as the tension preceding the contraction. The shortening velocity was defined as the radius shortening rate which is the slope of the radius-time curve for each contraction (arrows in Figure 7A). Since it turned out that the tension-shortening velocity relationship was a hyperbola similar to that described for skeletal muscle, Hill's equation can be used to characterize this relationship[5].

$$(T + a)(V + b) = (T\max + a)b$$

where T represents tension, V the velocity of shortening, Tmax the maximum tension at which the muscle is first unable to move, a and b are constants with dimensions of force and velocity, respectively. Figure 7A shows the bag radius (derived from the CSA) and the pressure during ramp distension of the duodenum. The slope of the contraction curve (arrows representing the contraction velocity) decrease as a function of the degree of distension. Force-velocity curves expressed as circumferential preload tension-radius shortening velocity diagrams are shown in Figure 7B for the duodenum in controls and systemic sclerosis patients. The velocity of shortening decreased with increasing preload tension. *T*max and the muscle power were lowest in the patients and association was found between the duration of the disease and the maximum stretch ratio. Decreased muscle function may explain the GI symptoms reported in this patient group and should be considered as an important factor in the diagnosis and treatment.

In conclusion, current understanding of the "mechanical" physiology of the GI tract is based on measurement techniques such as manometry. The development of new analysis of bag distention data will contribute significantly to the understanding of digestion in health and disease in the future. The impact of the proposed analysis is to better understand the muscle properties and how they relate to mechanism involved in symptom generation in organic and functional diseases in visceral hollow organs. Examples of frequent diseases of great interest are GERD, non-cardiac chest pain and functional dyspepsia. The perspective is that future treatment of GI disorders with affection of neuromuscular pathways can be mechanism- and evidence based with respect to such function curves.

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TOPIC HIGHLIGHT

Hans Gregersen, MD, *Series Editor*

Three-dimensional surface model analysis in the gastrointestinal tract

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Abstract

The biomechanical changes during functional loading and unloading of the human gastrointestinal (GI) tract are not fully understood. GI function is usually studied by introducing probes in the GI lumen. Computer modeling offers a promising alternative approach in this regard, with the additional ability to predict regional stresses and strains in inaccessible locations. The tension and stress distributions in the GI tract are related to distensibility (tension-strain relationship) and smooth muscle tone. More knowledge on the tension and stress on the GI tract are needed to improve diagnosis of patients with gastrointestinal disorders. A modeling framework that can be used to integrate the physiological, anatomical and medical knowledge of the GI system has recently been developed. The 3-D anatomical model was constructed from digital images using ultrasonography, computer tomography (CT) or magnetic resonance imaging (MRI). Different mathematical algorithms were developed for surface analysis based on thin-walled structure and the finite element method was applied for the mucosa-folded three layered esophageal model analysis. The tools may be useful for studying the geometry and biomechanical properties of these organs in health and disease. These studies will serve to test the structure-function hypothesis of geometrically complex organs.

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Key words: GI tract; Surface; Tension; Stress; Three-dimensional

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INTRODUCTION

Biomechanical changes due to functional loading and unloading of the human gastrointestinal (GI) tract are not fully understood. The GI function is usually studied by introducing probes in the GI lumen, e.g. for pressure recordings. Computer modeling based on medical imaging offers a promising alternative approach in this regard, with the additional ability to predict regional stresses and strains in inaccessible locations. Thus, the three-dimensional (3-D) geometry of visceral organs is of considerable interesting since the morphology and geometry are changed in various diseases[1,2]. The tension and stress distributions in the GI tract are related to distensibility (tension-strain relationship) and smooth muscle tone. The most widely used method for biomechanical stimulation of the gastrointestinal tract is bag distension[3,4]. Distension data are traditionally based on pressure-volume measurements[5-9]. Several studies suggest that the mechanoreceptors located in the gastrointestinal wall are tension receptors[10,11]. Distension technology has been refined by the introduction of the impedance planimetry technique and the endoscopic ultrasound probe[12-23]. These are now well-established methods for assessing circumferential strain, tension, stress and stiffness (slope of stress-strain relation). Previous GI studies have provided data on wall tension and stress in visceral organs. These studies, however, were based on two-dimensional models; i.e., the circumferential tension and stress have been determined at one specified position along the long axis of the organ. However, the tension distribution is related to the geometry (radii of curvature) and the distension pressure according to Laplace's law. Complex geometries will result in heterogeneous tension distributions. Therefore, three-dimensional geometry based on medical images is necessary for computing the tension and stress in the GI tract at various physiological and pathophysiological states.

Recently, 3-D surface model of the gall bladder, stomach, rectum and sigmoid colon have been developed by simultaneous cross-sectional imaging using ultrasonography, computer tomography (CT) or magnetic resonance

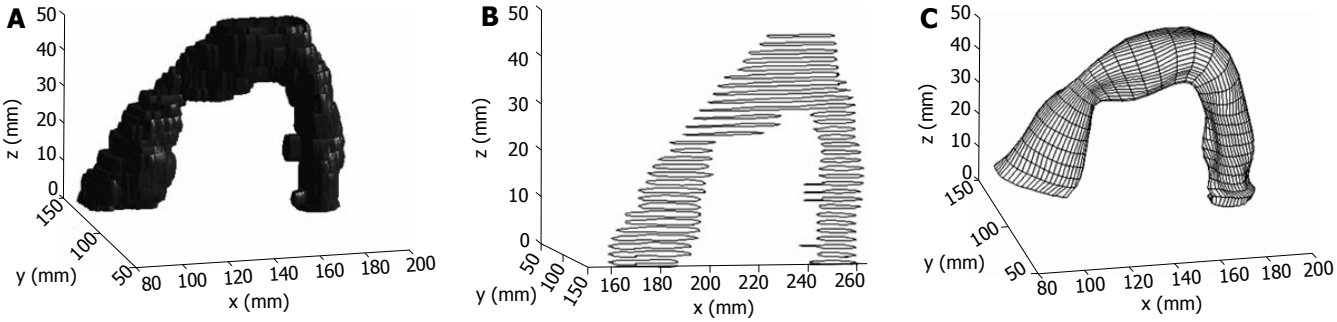

Figure 1 A 3-D sigmoid colon solid model (**A**), the surface model based on the cross sectional images (**B**)and the 3-D surface model with slices along the centre axis (**C**).

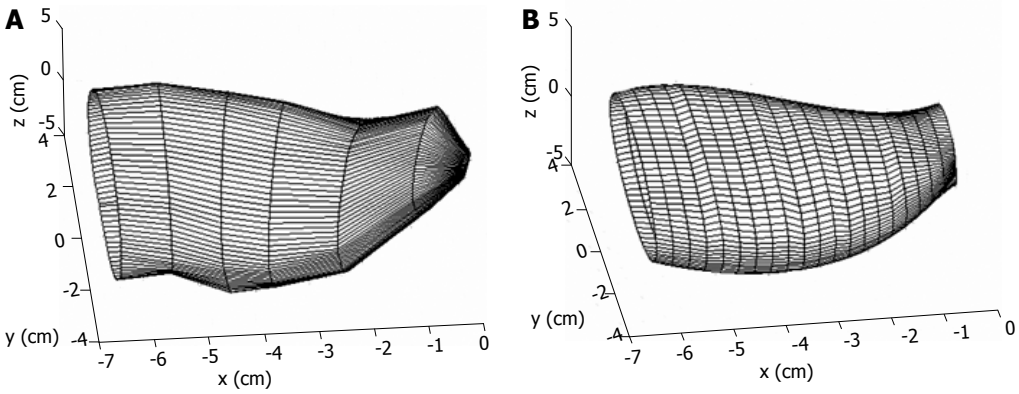

Figure 2 A 3-D reconstructed stomach antrum wall surface before (**A**) and after (**B**) surface smoothing by using the Fourier transform method.

imaging (MRI)) and pressure measurement during distension[24-27]. Using this method it is possible to model the 3-D geometry and mechanical properties of the gastrointestinal tract, including the spatial distribution of the 3-D principal curvatures, radii of curvature, wall thickness, tension and stress.

Examples of 3-D surface models of the GI organs, including stomach, rectum, sigmoid colon and gall bladder and the mucosal folded three layered esophageal finite element model are presented. Two different mathematic algorithms used for surface analysis were introduced. The human stomach[24] and gall bladder[25] models were generated based on the cross sectional images and the surfaces were analyzed theoretically by using a Fourier transform method. The human rectum[26], human sigmoid colon and rat stomach[27] were established from re-sliced images. The surfaces were analyzed locally by using a numerical method.

GEOMETRIC MODELS

Image acquisition and model reconstruction

The anatomical data used for the construction of the initial GI 3D models were obtained from ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI). The image processing method was used to identify the inner and outer wall boundaries and then a data cloud of a 3-D model was obtained. A 3-D sigmoid colon model built from cross sectional images is illustrated in Figure 1.

For GI organs such as the stomach[24], rectum[26] or sigmoid colon (Figure 1), the centre axis are curved. The distended organs are deformed along the curved axis. Alignment of data points along this curved centre axis was necessary to describe the GI model deformation. Based on a 3-D solid model generated from the cross sectional images, it can be re-sliced along the curved centre axis and the surface model based on the re-sliced images can be obtained. Figure 1 show a sigmoid colon solid model and a surface model generated from the re-sliced images.

Surface smoothing

The reconstructed surfaces have some irregularities due to the discretization of the images. The irregularities were removed using two different smoothing methods in our studies, one method was based on the Fourier transform algorithm and another one is a modified non-shrinking Gaussian smoothing method[28]. The two smoothing algorithms used in the surface analysis are illustrated in Appendix A and B. By using the algorithm described in appendix A, a complex surface can thus be described and smoothed by the surface function of Eq. A3. A smoothed stomach antrum model is illustrated in Figure 2. Figure 3 shows a sigmoid colon model that was smoothed based on the Gaussian smoothing method. With the continuous and smoothed surface, thus the surface can be approximated locally by a biquadric surface patch. The parametric surface used in our studies is a tensor product B-spline surface which gives the necessary continuity properties and with good surface approximation[29].

GEOMETRIC ANALYSIS

Curvature calculation

With the surface function Eq. (A3) or Eq.(B1) at the local co-ordinate system, the principle curvatures of

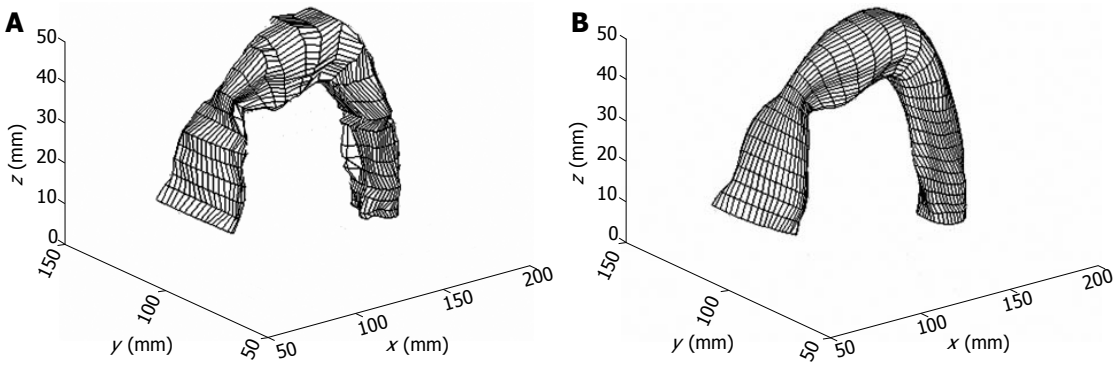

Figure 3 A 3-D sigmoid colon segment surface before (**A**) and after (**B**) surface smoothing by using the non-shrinking Gaussian method.

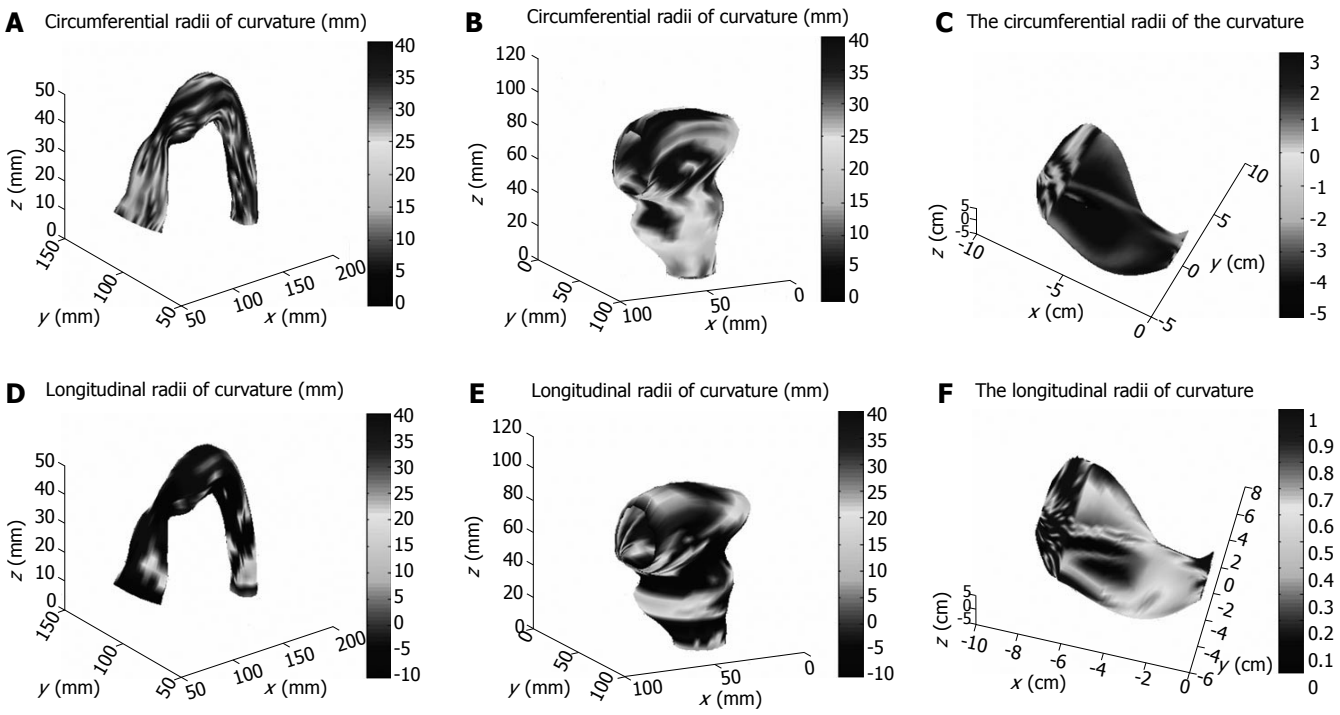

Figure 4 Circumferential (**A, B, C**) and longitudinal (**D, E, F**) radii of curvatures distributions in sigmoid colon (**A, D**), rectum (**B, E**) and human stomach (**C, F**).

the surface were generated from the coefficient of the first fundamental form (E,F and G) and the second fundamental form (L, M and N) of the surface function. The principal curvatures κ_1 and κ_2 can be combined from the *Gaussian* curvature (K_G) and the *Mean* curvature (K_M):

$$K_G = \kappa_1\kappa_2 = \frac{LN - M^2}{G - F^2} \quad (1)$$

$$K_M = \frac{1}{2}(\kappa_1 + \kappa_2) = \frac{1}{2}\frac{NE - 2MF + LG}{EG - F^2} \quad (2)$$

K_G is a particularly useful curvature parameter that indicates an elliptical surface ($K_G > 0$), a parabolic surface ($K_G = 0$) or a hyperbolic surface ($K_G < 0$).

Circumferential and longitudinal curvatures distribution on human sigmoid colon, human rectum and human stomach models are illustrated in Figure 4.

Tension and stress calculation

If we assume that the GI organs are thin-walled and its material properties are isotropic, the surface tension at a surface point is related to the transmural pressure by Laplace's equation as follows:

$$P = T\,[\kappa_1 + \kappa_2] \quad (3)$$

where κ_1 and κ_2 are the principal surface curvatures at a surface point. This equation is valid as long as the membrane is so thin that bending rigidity can be neglected and the curvature changes are not sudden. Accordingly, only the spatial points with positive Gaussian curvature were used for tension (and stress) calculations in this study. Stress is defined as the force acting on a unit area. The stress distribution on the surface can be calculated from wall tension and normal wall thickness as:

$$\sigma = T/h \quad (4)$$

T is the surface tension and h is the normal wall thickness. An example of tension and stress distribution on the rectum model are illustrated in Figure 5.

FINITE ELEMENT ANALYSIS FOR MUCOSAL FOLDED LAYRED ESOPHAGUS

The major shortcoming of the above surface analysis is the thin wall assumption. Thus only surfaces with positive principal curvatures in both directions could use Laplace's

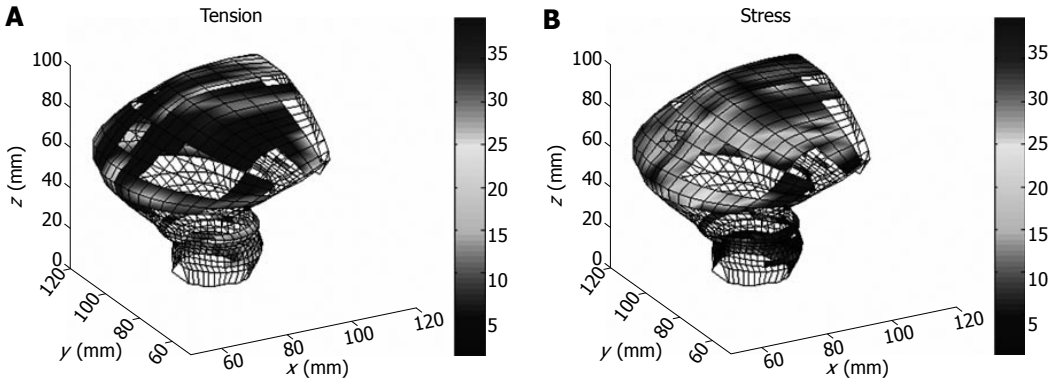

Figure 5 Tension (**A**) and stress (**B**) distribution on a distended rectum model.

equation for tension and stress calculation (Figure 5). To overcome this limitation, numerical methods such as finite element analysis must be adopted. The finite element method has been used for modeling GI tract bioelectric activity[30,31] and the layered esophagus distension simulation[32,33]. Figure 6 is a mucosal folded three layered esophageal finite element model. It is indicated that the stress distribution throughout the esophageal wall was not uniform and the stress was discontinuous between the muscle and mucosal-submucosal layer (Figure 6). Thus the Laplace's law is no longer valid in such layered and thick wall models.

PERSPECTIVES

Mechanosensitive receptors are important for GI tract function and sensation. The intraganglionic laminar nerve ending (IGLE) is believed to be a mechanosensitive receptor and is located within the smooth muscle-lined regions of the GI tract[34,35]. It is generally believed that they are tension receptors. The neurons respond in an intensity-dependent manner to the gastric distension[35,36]. Hence, determination of the stress or tension locally is important for understanding the mechanical environment at receptor sites. Methods based on pressure-volume measurements obtained during bag distension (such as the barostat) do not directly measure variables used in plane stress and strain analysis[5,8,12,22]. Because the bag can elongate, cross-sectional area cannot be derived from bag volume. Moreover, the main assumption for the computation of tension using the barostat and Laplace's law is that the geometry is spherical, although it seems obvious from medical imaging modalities such as ultrasound and magnetic resonance (MR)-scanning of the complex GI organs such as stomach or rectum that the assumption is not valid. Impedance planimetry measures luminal pressure and cross-sectional area in a short segment of hollow viscera during bag distension[14-18,22,37]. Based on these measures the circumferential strain, tension (applying Laplace law) and stiffness (slope of tension-strain relation) of a specific cross-sectional segment can be computed. This approach has limitations in irregular organs like the stomach, rectum or sigmoid colon because the calculated tension is based on the assumption of circularity, is only valid for thin walled structures, and only accounts for circumferential tension.

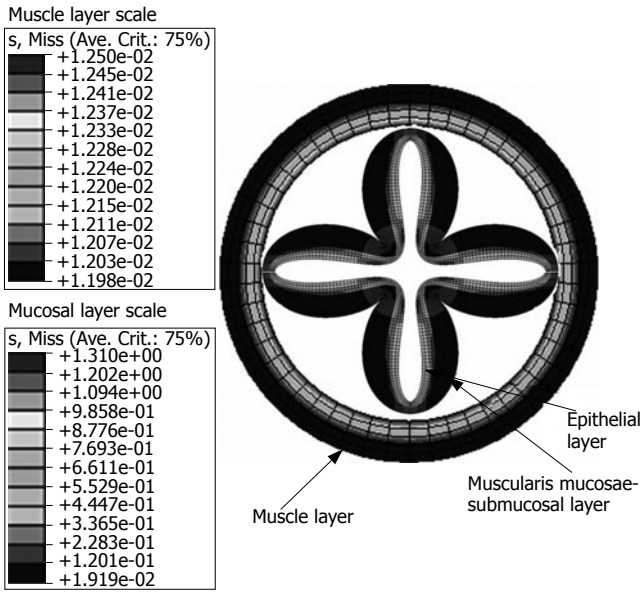

Figure 6 Mises stress (left) distribution in the mucosal folded three-layered esophagus at the bonded state.

In our 3D surface analysis, we generated the organ geometry based on 3-D ultrasound or MR images measurements. It is clear that GI organs such as the stomach, rectum, sigmoid colon and gall bladder have heterogeneous curvature distributions. Consequently, the geometry cannot be assumed spherical or cylindrical and that tension estimations based on Laplace's law are dubious because the geometry of the surface is complex. Two different methods were used for building 3D model and the surface approximation. By using the Fourier transform method the whole surface was reconstructed and smoothed simultaneously, i.e. the surface can be described theoretically and then the curvatures can be computed with good accuracy. However, it is difficult to describe all geometry characters in such complex GI organs by using only one function. Furthermore, the Fourier transform method can only be used on the surface generated from the horizontal cross sectional images. The analysis employed in these surface models produces large errors at both polar ends, especially to models with a curved center axis, such as the stomach, rectum or sigmoid colon. Since 3D solid model can be re-sliced in any direction, a new method for re-generating the 3-D surface

from the solid model was developed. By using the new method, all geometric characters can thus be kept in the surface model.

The major limitation of the surface analysis is the thin wall assumption. Thus only surfaces with positive principal curvatures in both directions could use Laplace's equation for tension and stress calculation. The finite element analysis or the finite difference method should be developed for further GI modeling analysis in the future. 3-D modeling based on cross-sectional imaging (CT, MRI and ultrasonography) has been used to characterize biomechanics and geometry in for instance the stomach[24,27], the rectum[26] cardiovascular system[38], and in computer-aided orthopedic surgery. The present analytical tool can be used to analyze complex structures for understanding biomechanical properties and visceral perception in other hollow visceral organs. The geometry and sensory aspects of diseases in the sigmoid colon (diverticular disease, irritable bowel syndrome, *etc.*), small bowel (motility disorders), stomach (motility disorders, non-ulcer dyspepsia, *etc.*) and esophagus (esophagitis, gastro-esophageal reflux disease, non-cardiac chest pain, *etc.*) can be obtained by applying a modified version of this method.

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APPENDIX A

Surface smoothing based on the Fourier transform method

Arbitrary point in a given cross section of the surface can be expressed in a polar co-ordinate system as (r, θ, z), where r, θ and z are the polar radius, angle and section height with respect to the origin. Each point can be fitted using a Fourier series as

Math1

$$r(\theta) = a_0 + \sum_{n=1}^{n=d} a_n \cos(n\theta) + \sum_{n=1}^{n=d} b_n \sin(n\theta) \qquad \text{(A1)}$$

where d denotes the degree of the Fourier series.

To approximate the Fourier coefficient as a function of the section height z, orthogonal polynomial were used as

$$a_i(z) = c_{0i} + c_{0i} z + c_{2i} z^2 + \cdots + c_{pi} z^p \qquad \text{(A2)}$$

where p is the degree of the polynomial and i is the index of the Fourier coefficient. The (r, θ, z) coordinates were described using vector rotation as

$$X(\theta, z) = (r(\theta, z)\cos(\theta), r(\theta, z)\sin(\theta), z) \qquad \text{(A3)}$$

where

Math2

$$r(\theta, z) = \begin{bmatrix} 1 \\ \cos\theta \\ \cdots \\ \sin d\theta \end{bmatrix}^T \begin{bmatrix} c_{00} & \cdots & c_{p0} \\ c_{01} & \cdots & c_{p1} \\ \cdots & \cdots & \cdots \\ c_{0(2d+1)} & \cdots & c_{p(2d+1)} \end{bmatrix} \begin{bmatrix} 1 \\ z \\ \cdots \\ z^p \end{bmatrix} \qquad \text{(A4)}$$

Hence, all the information of the surface is contained in the coefficient matrix, i.e., the surface was now transformed from a three-dimensional Cartesian coordinates (x, y, z) to a two-dimensional local tangent plane coordinates system (θ, z), where the θ is the tangent direction corresponding to the circumferential muscle direction and z to the longitudinal muscle direction, respectively.

APPENDIX B

Surface smoothing based on modified non-shrinking Gaussian method

This smoothing algorithm consists of two consecutive Gaussian smoothing steps with a positive scale factor λ applied to all the vertices of the shape, a second Gaussian smoothing step applied to all the vertices but with a negative scale factor μ, greater in magnitude than the first scale factor ($0 < \lambda < -\mu$). To produce a significant smoothing effect, these two steps must be repeated, alternating the positive and negative scale factors, a number of times. The relation between the position of the vertices of the surface before and after N iteration can be expressed as

$$X^N = ((I - \mu K)(I - \lambda K))^N X \qquad \text{(B1)}$$

where N was the number of iterations, λ and μ are two scale factors, I is the $n_V \times n_V$ identity matrix, $K = I - W$, W is the weight matrix and n_V is the number of the neighborhood of a vertex. In fact this method produces a low pass filter effect. The two scale factors determine the pass band and the stop band.

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REVIEW

Immune therapy including dendritic cell based therapy in chronic hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) infection is a global public health problem. Of the approximately 2 billion people who have been infected worldwide, more than 400 million are chronic carriers of HBV. Considerable numbers of chronic HBV carriers suffer from progressive liver diseases. In addition, all HBV carriers are permanent source of this virus. There is no curative therapy for chronic HBV carriers. Antiviral drugs are recommended for about 10% patients, however, these drugs are costly, have limited efficacy, and possess considerable side effects.

Recent studies have shown that immune responses of the host to the HBV are critically involved at every stage of chronic HBV infection: (1) These influence acquisition of chronic HBV carrier state, (2) They are important in the context of liver damages, (3) Recovery from chronic HBV-related liver diseases is dependent on nature and extent of HBV-specific immune responses. However, induction of adequate levels of HBV-specific immune responses in chronic HBV carriers is difficult. During the last one decade, hepatitis B vaccine has been administered to chronic HBV carriers as a therapeutic approach (vaccine therapy). The present regimen of vaccine therapy is safe and cheap, but not so effective. A dendritic cell-based therapeutic vaccine has recently been developed for treating chronic HBV infection. In this review, we will discuss about the concept, scientific logics, strategies and techniques of development of HBV-specific immune therapies including vaccine therapy and dendritic cell-based vaccine therapy for treating chronic HBV infection.

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Key words: Hepatitis B virus; Dendritic cells; Immune therapy; Vaccine therapy

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INTRODUCTION

Hepatitis B Virus (HBV) is a hepatotropic, noncytopathic, DNA virus which can cause acute as well as chronic infection. Chronic HBV infection remains a significant public health problem world-wide. It is estimated that there are at least 400 million HBV carriers in the world and that up to one million die annually due to HBV-associated liver diseases. Although the majority of the chronic HBV carriers may remain in an inactive phase associated with low viral replication and histological remission, a significant proportion will develop chronic hepatitis B (CHB), which is characterized by the presence of high viral replication, and chronic necroinflammation of the liver. Patients with CHB develop serious complications like liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Indeed, all chronic HBV carriers are at higher risk for HCC when compared with general population; the risk becomes much higher when LC develops. In addition, all chronic HBV carriers (inactive HBV carrier, patients with CHB [both treated and untreated], patients with LC and HCC) are permanent and living sources of HBV. Presence of million of chronic HBV carrier is practically responsible for the incidence of acute and fulminant hepatitis due to HBV[1].

No specific treatment is provided to 90% of chronic HBV carrier ('do nothing strategy'). Antiviral drugs are recommended only for patients with high HBV replication and necroinflammatory liver diseases. These drugs include type-1 interferons (IFN) and nucleosides analogues such as lamivudine and adefovir. It is difficult to assess the real therapeutic impact of antiviral drugs in chronic HBV carriers. On one hand, these drugs are recommended in some selected patients with CHB. On the other hand, the response varies considerably depending on virological and immunological statuses of the patients. Finally, many terms such as complete responder, biological responder, virological responder, transient responder, and sustained responder are used to assess responses to antiviral drugs. In general, antiviral drugs exhibit some beneficial effects in about 20%-30% of treated patients. However, IFNs

are costly and a significant proportion of patients with CHB are not eligible for treatment due to various reasons. On the other hand, lamivudine may need life-long usage. Treatment with lamivudine gives rise to emergence of mutant HBV and breakthrough hepatitis in considerable numbers of patients with CHB[2,3]. These problems related to currently available anti-viral therapies necessitate the development of alternative therapeutic approaches for chronic HBV carriers.

HBV is a non cytopathic virus and the immune responses of the hosts to HBV are important in different aspects of HBV infection. Although the cellular and molecular mechanisms underlying host/HBV interactions are not completely understood, various evidences suggest that host immune responses may have dual role in chronic HBV carriers. In one hand, liver damages in chronic HBV carriers are initiated and maintained by host immune responses. On the other hand, recovery from chronic HBV carrier state is dependent on the intensity and nature of host immune responses[4,5]. Accordingly, induction or suppression of generalized or polyclonal immune responses in HBV carriers is not likely to have therapeutic potentials. In line of this, it has been shown that although chronic HBV carriers exhibit impaired T helper 1 (Th1) responses, Th1-inducing cytokines (interleukin (IL)-12 and IFN-gamma) possess no notable therapeutic effects in these patients[6].

Only recently, some investigators have shown functional differences of HBV-specific immune responses and non HBV-specific immune responses in chronic HBV infection. It appears that products of non HBV-specific immune responses are responsible for liver damages. On the other hand, HBV-specific immune responses possess antiviral potentials without inducing liver damage in chronic HBV carriers[7,8].

Although this indicates that induction of HBV-specific immune responses in chronic HBV carriers may have therapeutic effect, it is extremely difficult to achieve this *in vivo*. Indeed, this is a confusing task. All chronic HBV carriers harbor HBV DNA and all HBV-related antigens. However, they do not exhibit adequate levels of HBV-specific immune responses. Initially, it has been assumed that chronic HBV carriers may be tolerant to some HBV-related antigens such as hepatitis B surface antigen (HBsAg). However, recent studies have revealed that HBsAg-specific immune responses are present in chronic HBV carriers, although the magnitude of immune responses may not adequate to clear or suppress the virus in chronic HBV carriers[9,10]

ROLE OF IMMUNE RESPONSES DURING ACQUISITION AND PROGRESSION OF CHRONIC HBV INFECTION AND ALSO DURING RECOVERY

Two billion people of the world exhibit either present or past infection with HBV and about 400 million of them are chronically infected with HBV. This indicates that less than 20% HBV-infected persons become chronic HBV carriers. It is natural to ask why some persons become chronic HBV carriers, whereas, others control the virus after an acute infection. Again, it is not clear why some chronic HBV carriers do not show any evidence of liver diseases, whereas, others suffer from progressive liver diseases like CH, LC and HCC. Finally, it is elusive why some chronic HBV carriers recover spontaneously. Although the exact cellular and molecular mechanisms underlying these are not completely clear, experimental evidences indicate that viral factor alone do not influence the courses of HBV diseases. Acquisition of HBV carrier state, degrees of liver damages in chronic HBV carriers and recovery from HBV carrier state are not dependent on any particular viral factor such as the amounts of the HBV, genotypes of the HBV, mutations at certain region of the HBV or expression of certain proteins of the HBV[1,2]. Recent investigations have revealed that immune responses of the hosts to HBV may be important in this context (Table 1). Vertical transmission of HBV from HBV-infected mothers (especially hepatitis B e antigen-positive) to neonates usually results in chronic HBV carrier state. This may be related to the concept of 'Neonatal Tolerance' as described by Burnett *et al*[11]. On the other hand, this may be due to impaired functional capacities of neonatal antigen-presenting cells[12].

Table 1 Role of immune responses in chronic HBV carrier

	HBV carriers	Possible causes
A: Acquisition of chronic State	A: Neonates of HBV-carrier mothers B: Immune suppressed Individuals	Inability to induce HBV-specific immunity
B: Induction and Progression of Hepatitis	Chronic hepatitis B	Accumulation of non-specific lymphocytes
C: Recovery from chronic HBV infection	A: Natural recovery	A: Strong and multispecific
	B: Acute hepatitis C: By antiviral drugs	HBV-related immune responses B: Antiviral therapy effective in immune modulatory phase

Some chronic HBV carriers never show any feature of liver diseases during their life time, although they harbor the HBV from their birth day. On the other hand, considerable numbers of chronic HBV carriers exhibit features of necroinflammatory liver diseases. Many of them also suffer from progressive liver diseases such as LC and HCC. HBV is a non-cytopathic virus and it is assumed that HBV-specific HLA-class restricted cytotoxic T lymphocytes (CTLs) act as the principle event determining the intensity of liver damages[13]. However, recent studies indicate that direct destruction of hepatocytes by the CTLs may not the only or principle mechanism of liver damages in patients with CHB. A series of investigations have shown that most of the liver-infiltrating lymphocytes are not HBV-specific. Liver injury could occur as a consequence of large infiltrate of antigen non-specific mononuclear cells. Different types of proinflammatory

cytokines such as IFN-gamma and tumor necrosis factoralpha may be responsible for induction of liver damages because neutralization of these cytokines prevent liver damages in various experimental models of hepatitis[14].

Host immune responses are also important during recovery from chronic HBV carrier state. The HBV-specific immune responses are strong and multispecific in patients with acute hepatitis B who control the virus replication after an acute infection. On the other hand, these are narrowly focused, weak and mono-specific in chronic HBV carriers who are unable to control the virus[13]. This indicates that if proper immune responses against the HBV are initiated after HBV infection, the patients are likely to recover. This is also manifested by the fact that antiviral drugs are mostly effective in immunodulatory phase, but not in immunetolerant phase, of chronic HBV carriers[15].

DEVELOPMENT OF EVIDENCE-BASED IMMUNE THERAPY FOR CHRONIC HBV INFECTION

As described above, immune responses of the hosts to HBV play important roles during acquisition of chronic HBV infection, during progression of liver damages in chronic HBV carriers and also for recovery from chronic HBV carrier state. In this context, it is natural ask what is meant by 'immune response of the host' in chronic HBV infection. This is a very complex issue and this review is not primarily intended to give a detail account of cellular and molecular events related to this. Here, we will focus on HBV-related 'immune responses' those are relevant for developing immune therapy for chronic HBV carriers.

All chronic HBV carriers studied to date, even the immune competent adults who acquire HBV infection, display infrequent, narrowly-focused, and weak HBV-specific immune responses[13]. The purpose of immune therapy in patients with chronic HBV infection is aimed at induction of strong HBV-specific immune responses *in vivo*.

It has been assumed that HBV-specific CTLs are primarily responsible for clearance of HBV-infected hepatocytes in chronic HBV carriers. CTLs are thought to destroy HBV-infected hepatocytes by cytolytic mechanisms. However, the overall concept regarding CTL and their role in destroying virus-infected cells has been changed not only in the context of HBV infection but also in other viral infections. First, CTL is not the only effector cells capable of destroying HBV-infected hepatocytes. Rather, other cells of both innate and adaptive immune system such as HBV-specific CD4 T cells, natural killer cells, macrophages, and neutrophils may also regulate replication of HBV and destruction of hepatocytes[14]. The next, CTLs are not only committed to destroy HBV-infected cells by directly interacting with hepatocytes. Rather, one of the major functions of CTL is to produce various types of cytokines. Series of experiments by different investigators have sown that cytokines are able to induce destruction of HBV or reduced replication of HBV by a non cytopathic mechanism[5,14,16].

Moreover, both HBV-specific and non HBV-specific immune responses are seen in patients with CHB. Several investigators have shown that HBV-specific immune responses are needed for control of HBV replication. On the other hand, non HBV-specific immune responses may induce destruction of hepatocytes. Patients with CHB express high levels of proinflammatory cytokines and accumulation of mononuclear cells in the liver. Recently, it has been shown that most of these mononuclear cells are non HBV-specific[17,18].

Taken together, these features indicate that induction of immune responses is not the main target of immune therapy for chronic HBV carriers. Rather, induction and maintenance of HBV-specific immune responses may have therapeutic implications. The challenging issue is to accomplish this in patients with chronic HBV infection.

VACCINE THERAPY: INDUCTION OF HBV-SPECIFIC IMMUNE RESPONSES

As an approach to induce HBV-specific immune responses in chronic HBV carriers, vaccines containing HBsAg and/or other HBV-related antigens have been administered in patients with CHB. Dienstag *et al* have used hepatitis B vaccine in patients with CHB in early 1980s, however, that study has not been regarded as vaccine therapy because the purpose of that investigation was to evaluate induction of anti-HBs in chronic HBV carriers[19]. The first clinical trial of vaccine therapy was done by Pol *et al* in 1994[20]. Subsequently, clinical trials of vaccine therapy have been performed during last one decade (Table 2)[21-27]. Most of these studies were pilot studies. The present regimen of vaccine therapy has been well tolerated in patients with CHB. It has caused reduced HBV replication, seroconversion to anti-HBe and normalization of serum transaminases in some patients with CHB. However, there is no standard protocol of vaccine therapy. Vaccine therapy has mainly been conducted as pilot studies. All investigators have used their own protocol to perform vaccine therapy. Variable doses of HBsAg (10-20 micrograms) have been used. In some clinical trials, only HBsAg-containing vaccines were used, whereas, in others vaccines containing HBsAg plus preS1 and preS2 proteins were used. The numbers of immunization also varied among studies (3-12 times). Most importantly, there are no standard criteria to assess the response of vaccine therapy.

CAN VACCINE THERAPY STAND THE TASTE OF TIME?

The present regimen of vaccine therapy is unlikely to be an independent therapeutic approach for patients with CHB. A lot of works must be done regarding vaccine therapy. There should be randomized controlled trials of vaccine therapy in different countries. More studies are needed regarding optimum doses of vaccine, compositions of vaccine and numbers of immunization. This therapy is safe for human usage. No side effects of this therapy have yet been published. Vaccines are commercially available and comparatively cheaper. At present, therapeutic efficacy of vaccine therapy is not so satisfactory. But, that is not so important if we consider its future potentials. The concept

of vaccine therapy is unique. Treatment of chronic infection by inducing antigen-specific immune responses deserves attention. There are ample opportunities to improve the present regimen of vaccine therapy.

At present, it appears that the present regimen of vaccine therapy can be used in some specific patients with CHB such as in patients with anti-HBe positive CHB and patients with low levels of HBV replication. The next, vaccine therapy can be used in combination with other antiviral agents. Vaccine therapy can be started after using antiviral drugs for some times in patients with CHB. This may allow better action of this therapy due to decrease of HBV load by antiviral drugs. Again, vaccine therapy can be used first and then antiviral agents can be applied after induction of host immune responses by vaccine therapy. This may be manifested in better therapeutic potential of antiviral agents in CHB patients. We have recently reported a combination therapy of lamivudine and vaccine therapy in patients with CHB. All patients receiving combination therapy became negative for HBV DNA in the sera, whereas, only 56% patients with lamivudine monotherapy lost HBV DNA in the sera within 12 mo. Also, patients with combination therapy did not develop HBV DNA mutation or breakthrough hepatitis[28].

Table 2 Vaccine therapy in patients with chronic hepatitis B. The criteria of response to this therapy have been determined by individual investigators

Protocol	Response	Reference
HBsAg/pre S2, 3 times	Responders: 12 of 32 (38%)	Pol *et al*[20]
HBsAg/anti-HBs vaccine; 3 times	Responders: 9 of 15 (60%)	Wen *et al*[21]
PreS2/S, 3 times (controlled trial)	Responders: 18.8%	Pol *et al*[22]
HBsAg; 12 times	Responders (1 yr: 55%, 2 yr: 77%)	Horiike *et al*[23]
PreS2 vaccine; 3 times	Responders: 60%	Senturk *et al*[24]
S/pre S2; 6 times	Decreased HBV DNA	Ren *et al*[25]
HBsAg/preS2; 3 times	Immune tolerant phage: no significant change	Dikici *et al*[26]
PreS2; 3 times	anti-HBs; 3 of 31 cases	Yalcin *et al*[27]
Combination of lamivudine and HB vaccine	HBV DNA negativation in all 15 cases at 1 yr	Horiike *et al*[28]

DEVELOPMENT OF NEXT GENERATION VACCINE FOR TREATMENT OF CHRONIC HBV INFECTION

Vaccine therapy in patients with CHB was started without adequate discussions regarding its scientific validity and utility. Vaccine therapy was done without paying full attention to common consensuses of immunology and virology. It was surprising to most clinicians why administration of vaccine containing HBsAg in CHB patients will have therapeutic effect because all CHB patients have abundant amounts of HBsAg in the sera and the liver. Moreover, it has been assumed that HBsAg is a tolerogenic antigen in CHB patients. Also the mechanism of action of vaccine therapy in patients with CHB has not been properly analyzed. In general, new therapeutic approaches are usually tested in animal models of human diseases and then these are applied in patients. Vaccine therapy is an exceptional type of therapy. It was first done in patients with CHB without performing preclinical trials in animal models of chronic HBV infection.

In fact, there has been no good animal model of chronic HBV infection. Natural infection of the HBV is limited to some animals only. Lack of an animal model was a major limitation to study immune pathogenesis of chronic HBV infection. During 1980s, HBV transgenic mice (HBV-TM) were produced by microinjecting HBV genome into fertilized eggs of mice[29]. Naturally, there are many differences between HBV-TM and patients with CHB, but HBV-TM represent a suitable immunological animal model of HBV infection. We and others used HBV-TM to develop insights about HBV immunopathogenesis to study various cellular events those can not be investigated directly in human[29,30].

We used a line of HBV-TM that expressed HBsAg and HBV DNA in the sera. This HBV-TM also expressed HBV-related mRNAs in the liver[31]. HBV-TM did not express antibody to HBsAg (anti-HBs) and HBsAg-specific lymphocytes were not detected in HBV-TM. We showed that HBV-TM were unresponsiveness to HBsAg due to impaired function of antigen-presenting dendritic cells (DCs)[32]. Moreover, the immune response defects of HBV-TM were over come by activating endogenous DCs of HBV-TM[33,34]. Next, four controlled trials of vaccine therapy were conducted in HBV-TM. HBV-TM were immunized with vaccine containing HBsAg once in a month for 12 times. It was possible to induce both HBsAg-specific humoral and cellular immune responses in HBV-TM by administration of vaccine containing HBsAg[35-38]. Study on the mechanism of action of vaccine therapy revealed that vaccine therapy induced HBsAg-specific immune responses by activating autologous DCs[37]. Also, the functions of DCs prior to start of therapy had prognostic value during vaccine therapy[38].

DEVELOPMENT OF HBSAG-PULSED DC

DCs are essential for induction and maintenance of antigen-specific specific immune responses. DCs recognize antigens at the site of their localization. They internalize antigens and process them at their endosomal compartments. This leads to expression of antigenic peptides at the surface of DCs. These DCs migrate to lymphoid tissues and activate lymphocytes to become either CTL or CD4+ T cells or antibody-producing B cells[39,40].

During vaccine therapy in HBV-TM, vaccine containing HBsAg was administered to HBV carriers to induce HBsAg-specific immune responses. This was done with the assumption that DCs of HBV-TM will recognize injected HBsAg. Subsequently, HBsAg would be captured, internalized, and processed by DCs of HBV-TM. It was assumed that DCs would activate lymphocytes to induce HBsAg-specific immune responses (Figure 1). But, DCs of HBV-TM had impaired functional capacities and thus endogenous DCs of HBV-TM are not potent inducer of HBsAg-specific immune responses.

We postulated that a powerful regimen of vaccine therapy can be developed by loading DCs with HBsAg in vitro (HBsAg-pulsed DCs). If HBsAg-pulsed DCs exhibit markers of activation and maturation, HBsAg-pulsed DCs will be able to directly induce HBsAg-specific immune responses without any influence of endogenous DCs of chronic HBV carriers. Accordingly, we cultured murine spleen DCs and HBsAg together to produce HBsAg-pulsed DCs. HBsAg-pulsed DCs were more immunogenic than unpulsed DCs. Only two injections of HBsAg-pulsed DCs caused negativity of HBsAg from HBV-TM (Figure 2). HBV-TM injected with HBsAg-pulsed DCs also developed anti-HBs in the sera[41]. Administration of HBsAg-pulsed DCs also induced anti-HBs in immunosuppressed HBV-TM [42, 43].

TRANSLATION OF IMMUNOLOGICAL KNOWLEDGE FROM THE BENCHES TO PATIENT'S BEDSIDES

Wang *et al.* have first reported about functional impairment of DCs in patients with CHB[44]. Subsequently, we localized HBV DNA and HBV RNA in human blood DCs and also showed low IL-12 production capacity of DCs from CHB patients[45]. Similar data regarding functional defects of DCs of chronic HBV carriers have been reported by other investigators during the last 3 year[46,47]. These studies provided the scientific basis for developing DC-based therapy for patients with CHB.

We followed a very careful protocol to produce HBsAg-pulsed blood DCs for human usage[48]. DCs were enriched from human peripheral blood and cultured with HBsAg to prepare HBsAg-pulsed DCs. We used commercial vaccine as a source of HBsAg. The functional capacities of HBsAg-pulsed DCs were assessed *in vitro*. Next, HBsAg-pulsed DCs were administered to human volunteers. There was no features of inflammation, abnormal liver and kidney functions in any volunteers. No volunteer showed any features of autoantibody or autoimmune diseases. Administration of HBsAg-pulsed DCs induced anti-HBs antibody in all volunteers, although the levels of antibody varied among volunteers.

Then, a study was conducted to assess the safety of HBsAg-pulsed DCs in patients with CHB. We prepared DCs from peripheral blood of patients with CHB. Human blood DCs were cultured with HBsAg in commercial vaccine to prepare HBsAg-pulsed DCs. HBsAg-pulsed DCs were administered to patients with CHB. No adverse effects were detected in these patients. Recently, a group of researcher from China has used HBsAg-pulsed DCs for treating patients with CHB[49]. The data of their study is highly encouraging because HBsAg-pulsed DCs exhibited both antiviral and immune modulatory capacities in some patients with CHB. Moreover, they have reported that patients of CHB with normal transaminase levels may also be benefited from this therapy. Although the data of this study is highly stimulating, more information is necessary about the source of HBsAg and characterization of HBsAg-pulsed DCs. Also, detailed data about study protocol will allow undertaking more clinical trials of these therapeutic approaches.

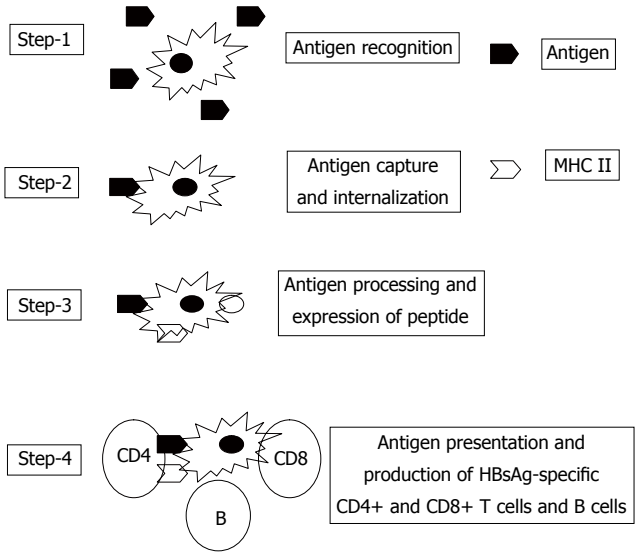

Figure 1 DC/antigen interactions have been shown in 4 steps. Three of these steps are accomplished *in vitro* (step 1-3) when HBsAg-pulsed DCs are prepared.

PRESENT AND FUTURE OF IMMUNE THERAPY AGAINST CHRONIC HBV INFECTION WITH AN EMPHASIS OF IT'S UTILITY IN DEVELOPING NATIONS

The life cycle of HBV, presence of cccDNA of HBV at hepatocytes, and existence of HBV at different extrahepatic sites are major obstacles for eliminating this virus from chronic HBV carriers. It is now apparent that complete eradication of the HBV from chronic HBV carriers may be an unachievable goal[2].

It is also evident that there should be different strategies of HBV control program in developed and developing nations of the world. Less than 10% of total HBV carriers live in developed countries. Moreover, both vertical and horizontal transmissions of HBV have effectively been controlled in most of these countries.

On the other hand, the health care delivery system and economic conditions of developing nations are completely different from those of developed nations, where more than 90% chronic HBV carriers reside. The public health measures are not well developed in most developing nations. Contaminated needles and syringes are used in many facilities, especially in rural areas. There are many chronic HBV carriers among professional blood donors. The numbers of drug abusers are increasing in many of these courtiers. Thus, the numbers of HBV carriers will remain unchanged or increase in most developing nations. China alone harbors about 130 million HBV carriers. Also, there are several million HBV carriers in India.

Regarding treatment, the medical infrastructures of most developed nations allow usage of costly antiviral drugs, regular follow up of treated patients, proper handling of complications and adequate approaches to fight against the mutant HBV. Newer nucleoside analogues are coming to the market. In future, combination therapy with different nucleoside analogues will mainly be used

in patients with CHB in most developed countries. In developed countries, national liver and gastroenterology associations update therapeutic regimens for chronic HBV infection from time to time.

However, usage of antiviral drugs in patients with chronic HBV infection and proper follow up of these patients are difficult in developing nations. IFNs can be afforded by a very small percentage of patients with CHB in these countries. Most of them are not aware of limitation of antiviral drugs in chronic HBV infection. It is generally told that antiviral drugs are effective in about 30% patients with CHB. However, antiviral drugs are recommended for only 10% patients with chronic HBV infection. These drugs are not recommended for majority of chronic HBV carriers. In most developing nations, there is no appropriate system to follow emergence of mutant HBV following lamivudine therapy.

Vaccine therapy in patients with CHB was first conducted in 1994. However, few randomized controlled trials of vaccine therapy have been conducted till now. This situation is different regarding clinical trials of antiviral drugs in developed countries. Antiviral drugs are developed by multinational companies. In contrary, vaccine therapy for chronic HBV carriers has been developed by scientists. This is a cheap therapeutic option for patients with CHB. It is also safe. Vaccine therapy may be popularized if data of controlled trials show their real potentialities. There should be randomized controlled trials of vaccine therapy in patients with CHB in developing countries.

One of the main purposes of immune therapy is to achieve what antiviral drugs could not accomplish in most chronic HBV carriers. The potentiality of vaccine therapy for treating chronic HBV carriers lies in its concept, not in its present ways of application. Truly speaking, this therapy is still in its infancy. Several clinical and experimental studies should be done to optimize protocols of vaccine therapy. At present, commercial vaccines contain HBsAg and/or other components of surface antigens such as preS and preS2 antigens are used in vaccine therapy against chronic HBV carriers. Vaccines containing hepatitis B core antigen or polymerase antigen can also be used in future for treating patients with CHB. However, the main problem is to get safe and human consumable forms of these antigens.

Till now, antigen-pulsed DCs have been used almost exclusively in patients with cancers. Although there are ample opportunities of using antigen-pulsed DCs in chronic infection, allergy and autoimmunity, very few investigators have tried to materialize this in nonmalignant patients. However, we have learnt many important lesions from DC-based therapy in patients with cancers. In the context of DC-based therapy, there is a need to get proper antigens. The next is about the method of preparation of immunogenic antigen-pulsed DCs. Culture of DCs and antigens may lead to production of immunogenic as well as tolerogenic DCs. Accordingly, it is needed to check the nature of antigen-pulsed DCs before administration those to patients. Again, safety is a major concern when antigen-pulsed DCs are administered to patients with chronic HBV infection. In contrast to patients with cancers, patients with chronic HBV infection are immune competent. Administration of antigen-pulsed DCs may induce autoimmunity in these patients.

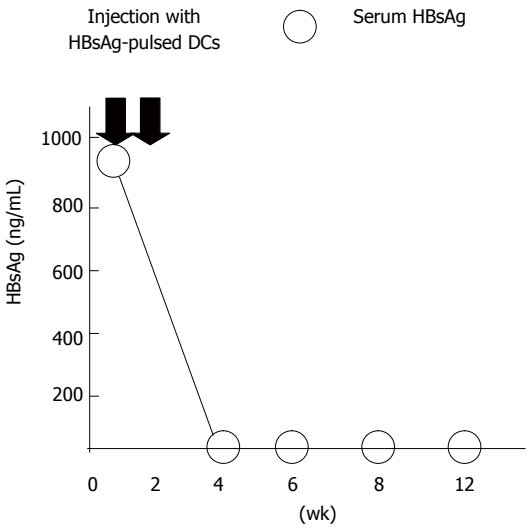

Figure 2 Potent immune modulatory capacity of HBsAg-pulsed DCs in HBV transgenic mice. HBV transgenic mice expressed HBsAg in the sera but not anti-HBs. After two injections of HBsAg-pulsed DCs, these mice became negative for HBsAg in the sera.

At present, clinical trials have been conducting with HBsAg-pulsed DCs. In future, hepatitis B core antigen-pulsed DCs may be used in patients with CHB. The major limitation is to get human grade HBV-related antigens. We used commercial vaccine containing HBsAg to pulse DCs with HBsAg. Chen *et al*[49] have used HBsAg-pulsed DCs in patients with CHB, but they have not mention about the source of HBsAg. The availability of GMP standard HBsAg and other HBV-related antigens will contribute significantly for conducting more clinical trials with antigen-pulsed DCs in patients with CHB. A major challenge will be to achieve GMP standard in developing nations for conducting cell-based therapies including DC-based therapy. This issue can be solved by establishing scientific collaboration among developing countries, developed countries and international agency like World Health Organization.

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REVIEW

Non-steroidal anti-inflammatory drugs in prevention of gastric cancer

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) including cyclooxygenase 2 (COX-2) selective inhibitors, are potential agents for the chemoprevention of gastric cancer. Epidemiological and experimental studies have shown that NSAID use is associated with a reduced risk of gastric cancer although many questions remain unanswered such as the optimal dose and duration of treatment. The possible mechanisms for the suppressor effect of NSAIDs on carcinogenesis are the ability to induce apoptosis in epithelial cells and regulation of angiogenesis. Both COX-dependent and COX-independent pathways have a role in the biological activity of NSAIDs. Knowledge of how NSAIDs prevent neoplastic growth will greatly aid the design of better chemopreventive drugs and novel treatments for gastric cancer.

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Key Words: Nonsteroidal anti-inflammatory drugs; Gastric cancer; Cyclooxygenase; Prevention; Intervention

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INTRODUCTION

Gastric cancer is a major cause of death in the world. However, the etiology of gastric cancer is not fully understood. Although early diagnosis and treatment of gastric cancer significantly improve prognosis[1,2], the 5-year survival rate is only 10%-15% in those with advanced disease[3]. Therefore, the primary prevention of gastric cancer is of particular importance. Several studies have shown that human gastric carcinoma expresses significantly higher levels of cyclooxygenase (COX)-2 than non-cancerous adjacent mucosa[4], suggesting that COX-2 plays a possible role in gastric carcinogenesis. Numerous observations and clinical studies have suggested that the use of NSAIDs is associated with reduced incidence of gastric cancer[5-8]. NSAIDs, particularly the highly selective COX-2 inhibitors, are promising chemopreventive agents[9-12].

Although the effects of NSAIDs on gastric cancer remain unclear, both COX-dependent and independent mechanisms may have roles in the biological activity of NSAIDs. In this review, we concentrate on the use of NSAIDs for gastric cancer prevention and the underlying mechanisms. Furthermore, we also suggest research opportunities on clinical application of NSAIDs for gastric cancer prevention.

NSAIDS AND GASTRIC CANCER

Epidemiological evidence

There are indications, although still limited and preliminary, of a protective effect of NSAIDs on gastric cancers. The risk of gastric cancer is reduced, though not significantly, in the Swedish[13] cohorts of patients with rheumatoid arthritis, with the relative risk (RR) of 0.63 (95%CI = 0.5 to 0.9) for NSAID users. In the American Cancer Society cohort, there is a significant reduction in the risk of death for gastric cancer in aspirin users (RR = 0.64, 95%CI = 0.51 to 0.80). In addition, a dose-dependent trend in risk reduction has been observed, with the RR of 0.73 (95%CI = 0.56 to 0.96) for occasional use (1-15times per month) and 0.49 (95%CI = 0.33 to 0.74) for use 16 or more times per month[5]. With regard to case-control studies, in an American study of cancers of the esophagus and stomach, based on 261 adenocarcinomas of the cardia, 368 noncardia and 695 population controls, current aspirin users have a lower risk for noncardia cancer (RR = 0.46, 95%CI = 0.31 to 0.68), but not for cardia cancer (RR = 0.80, 95%CI = 0.54 to 1.19) when compared to never users. However, there is no significant trend for the risk reduction of gastric cancer with the frequency and the duration of aspirin use[6]. A case-control study from Russia was conducted on 448 gastric cancer cases and 610 hospital controls. The use of aspirin is significantly associated with a risk reduction for the development of gastric cancer (RR = 0.49, 95%CI = 0.31 to 0.77), although the favorable effect seems to be limited for patients with noncardia cancer and *Helicobacter pylori* (*H pylori*) infection[7]. Another case-control study from Sweden, conducted on 567 cases

and 1165 hospital controls, reported that users of aspirin have a moderately reduced risk of gastric cancer compared to never users (RR = 0.7, 95% CI: 0.6 to 1.0), and gastric cancer risk falls with the increasing frequency of aspirin use. Moreover, the risk reduction is statistically significant not only for cardia but also for noncardia cancer, and is slightly more marked in patients with *H pylori* infection[8].

However, there are several limitations for the above observational studies. First, the small number of cases may result in inadequate statistical power[7,8,15]. Second, the choice of control subjects in case-control studies may distort the results because hospital-based controls can not represent population-based controls[16]. Third, different definition of drug exposure and different doses of drugs have been evaluated in these studies. Therefore, the optimal duration and dose of treatment could not be recommended from these studies.

A recent systematic review with meta-analysis of nine observational studies evaluating the association between NSAID use and the risk of gastric cancer suggested that long-term use of aspirin or other NSAIDs is associated with a statistically significant, dose-dependent reduction in the risk of gastric cancer (OR = 0.78, 95%CI = 0.69 to 0.87). When the analysis is stratified by the site of gastric cancer, use of NSAIDs is associated with a statistically significant lower risk for noncardia gastric cancer (OR = 0.72, 95%CI = 0.58 to 0.89), but not for gastric cancer at the cardia. However, whether the minimum effective duration of NSAID use can reduce gastric cancer risk remains undetermined because of the relatively small number of studies, the use of different cut-off time points of treatment duration, and varying definitions of drug exposure[14]. More prospective studies are needed to clarify whether a duration effect exists in the relationship between NSAID use and gastric cancer risk.

Animal models and in vitro studies

The results obtained from animal models evaluating the effect of NSAIDs on gastric cancer have been conflicting. Two studies on animal models of carcinogens suggested that NSAIDs (aspirin and flurbiprofen) could enhance chemical-induced gastric carcinogenesis in rats[17,18], indicating that prostglandins (PGs) produced by the gastric mucosa protect against the action of carcinogens in this rodent model. In contrast, broad anticarcinogenic effects of NSAIDs have been demonstrated both *in vivo* using laboratory animals and *in vitro* using cancer cell lines. Chemically-induced gastric tumors can be reduced by aspirin[19], indomethacin[20], sulindac and ibuprofen[21] in mice. Sawaoka *et al*[22] have investigated the effects of NS-398, a selective COX-2 inhibitor, or indomethacin, a non-selective COX-2 inhibitor, on the growth of gastric cancer xenografts *in vivo*. Both drugs could reduce the tumor volume significantly in a dose-dependent manner. Fu *et al*[23] also found that sulindac and celecoxib obviously decrease the blood vessel quantity and tumor size of SGC7901 cancer xenografts. In a recent study Hu *et al*[24] showed that treatment with celecoxib could suppress MNNG-induced gastric cancer in rats. Indomethacin, sulindac and NS-398 significantly inhibit the proliferation and growth of human gastric cancer cell line MKN28 *in vitro*[25,26].

POSSIBLE MECHANISMS OF CANCER PREVENTION

The results described above provide convincing evidence that non-selective NSAIDs and selective COX-2 inhibitors can inhibit gastric cancer cell formation and growth. However, the molecular basis for the striking chemo-preventive effects has not been fully clarified. Several studies indicate that COX-2 expression is a relatively early event during carcinogenesis in the stomach[27-30]. The best known target of NSAIDs is COX, the rate limiting enzyme in the conversion of arachidonic acid to prostanoids. Thus, the chemo-preventive effects of NSAIDs on gastric cancer may be attributed to inhibition of COX and the resulting decrease in production of PGs[31-33]. However, there are several studies that conflict with this observation. For example, NSAID derivates that lack the ability to inhibit COX (sulindac sulfone) have been shown to inhibit gastrointestinal tumor growth *in vivo* and *in vitro*[34-37]. COX-1 and COX-2 null mouse embryofibroblast cells remain sensitive to the anti-proliferative and anti-neoplastic effects of NSAIDs[38]. Furthermore, the concentration of NSAIDs that inhibits the growth of cancer cells *in vitro* is 10-100 times higher than that required to inhibit COX activity, suggesting the existence of additional cellular targets.

Induction of apoptosis

A substantial body of evidence now supports the idea that the induction of programmed cell death (apoptosis) is one of the main ways by which NSAIDs prevent cancer in a COX-2 dependent manner or a COX-2 independent manner.

COX 2-dependent mechanisms

One of the primary pharmacological properties of the NSAIDs is their ability to inhibit the COX. Analysis of COX expression has shown that COX-2 is increased in gastric cancer, but not in normal mucosa[4,39-42]. These findings support the idea that COX-2 over-expression is important during gastric carcinogenesis. In addition, over-expression of COX-2 can increase production of PGE2, adhesion to the extra-cellular matrix, concentrations of BCL2, and reduce TGF-2 receptor expression and E-cadherin protein. All these changes, which suggest an increased tumorigenic potential, support the notion that COX-2 overexpression alters the biology of cells and plays a part in the transformation process[43,44]. The question of how COX-2 inhibition induces apoptosis has been hotly debated. Several studies have suggested that decreased COX-2 activity can reduce eicosanoids such as the PGs, and in turn affect cell proliferation and apoptosis[45]. So far, there is no definitive evidence that supports the existence of a signaling pathway through which PGs can directly affect apoptosis. However, specific observations provide some clues and warrant mention here. The peroxisome proliferator-activated receptors (PPARs), a family of ligand-activated transcription factors comprising a subset within the steroid receptor family of intracellular receptors, have been shown to modulate the apoptotic response of various cell types[46,47]. It is conceivable that certain COX-2

products may be physiological ligands for the PPARs. Thus, it is hypothesized that PPARs are downstream of COX-2 in gastric carcinogenesis and may play a part in NSAIDs-mediated antineoplastic effects[48-50].

Arachidonic acid may also provide a mechanism underlying COX-2-dependent NSAIDs-induced apoptosis. Arachidonic acid is produced by hydrolysis of phospholipids and triglycerides. The phospholipase A2 cleaves phospholipids and releases arachidonic acid into the cytosol. Once released, it serves as a precursor for PGs production and is a signaling molecule in its own right. Treatment with various NSAIDs results in inhibition of COX-2 and a dramatic increase in the concentration of arachidonic acid, the main substrate for COX-2[45,51]. This buildup of arachidonic acid stimulates the sphingomyelinase to convert sphingomyelin to ceramide, a potent inducer of apoptosis[52-55]. Other studies have shown that the cellular concentration of arachidonic acid is a general mechanism by which apoptosis is regulated[56-59]. It seems that arachidonic acid can alter mitochondrial permeability and cause cytochrome C release, leading to apoptosis[58,60].

COX 2-independent mechanisms

Several recent observations cast doubt on the idea that COX is the sole target of NSAID action. The finding that some NSAIDs can inhibit proliferation and induce cell death in cells that do not express COX, suggests that other targets may play a part in NSAIDs-mediated apoptosis[61].

One potential mechanism involves the transcription factor NF-κB, which promotes cell survival and enhances proliferation. Several investigators have suggested that NSAIDs could promote apoptosis by inhibiting NF-κB signaling pathway[62-64]. The effect that seems to be due to its ability to inhibit the activity of IκB kinase β (IKKβ), an enzyme that activates the NF-κB pathway by phosphorylating the inhibitory subunit of NF-κB (IκB-α) and targeting it for destruction. Subsequently, genes required for cancer cell growth and survival may not be transcribed[65]. Furthermore, Yasui *et al*[66] reported that sulindac inhibits TNF-α-mediated NF-κB activation and greatly sensitizes human gastric cancer cell line MKN45 to TNF-α. The study data strongly suggest that combination therapy of TNF-alpha with sulindac may sensitize tumor cells to TNF-α and augment its proapoptotic potential. Moreover, Wong *et al*[67] showed that SC236, a selective COX-2 inhibitor, could inhibit NF-κB mediated gene transcription and binding activity in gastric cancer. This effect occurs through a mechanism independent of COX activity and PG synthesis. Furthermore, unlike aspirin, SC236 affects neither the phosphorylation, degradation, nor the expression of IκB-α, suggesting that the effects of SC236 are independent of IKK activity and IκB-α gene transcription. Instead, SC236 works directly through suppressing the nuclear translocation of RelA/p65. It is possible that SC236 directly targets proteins that facilitate the nuclear translocation of NF-κB[67].

The role of protein kinase C (PKC) and apoptosis-related oncogenes has also attracted more attention. Zhu *et al*[68] showed that both aspirin and indomethacin induce apoptosis in gastric cancer cells which may be mediated by up-regulation of *c-myc* proto-oncogene. PKC activation can abrogate the effects of NSAIDs by decreasing *c-myc* expression. Jiang *et al*[69] also showed that SC-236 induces apoptosis in AGS cells. SC-236 down-regulates the protein expression and kinase activity of PKC-β(1) in AGS cells, which provides an explanation for COX-independent apoptotic effects of selective COX-2 inhibitors in cultured gastric cancer cells. They also suggested that PKC-β(1) acts as a survival mediator in gastric cancer. Down-regulation of PKC-β(1) by SC-236 may provide new targets for future treatment of gastric cancer[69].

Furthermore, it was reported that the pro-apoptotic proteins Bax and Bak play a role in NSAIDs-induced gastric cancer cell apoptosis[70], suggesting that one of the major pathways mediating the anti-tumor response of aspirin and indomethacin in gastric cancer cells is through the up-regulation of Bax and Bak and activation of caspase-3.

Besides, Wu *et al*[71] demonstrated that SC-236 induces apoptosis in gastric cancer cells via the up-regulation of 15-lipoxygenase-1 (15-LOX-1), and increases endogenous 13-S-hydroxyoctadecadienoic acid (13-S-HODE), a novel target for chemoprevention effect of NSAIDs.

Regulation of angiogenesis

It is now clear that angiogenesis is critical for the progression of most human cancers[72]. Angiogenesis is a complex process controlled by a balance of angiogenic and angiostatic factors involved in multiple pathways that result in endothelial cell proliferation, differentiation, and organization into a functional network of vascular channels. Among the reported angiogenic factors, vascular endothelial growth factor (VEGF) is the most powerful endothelial cell specific mitogen that plays a key role in the complicated process of angiogenesis.

COX-2 has been shown to directly promote angiogenesis in several different experimental systems[73,74]. Masferrer *et al*[80] have examined more than 150 samples of different types of human cancers and found that COX-2 is present in the angiogenic vasculature in most of the tumors analyzed. Therefore, COX-2-derived PGs contribute to tumor growth by inducing newly formed blood vessels that sustain tumor cell viability and growth. Furthermore, xenografts of COX-2 over-expressing gastric cancer, when implanted into nude mice, express angiogenic factors in a COX-2-dependent manner, and both angiogenesis and tumor growth can be inhibited in these mice by administration of NSAIDs[23,78].

COX-2 expression has been shown to correlate with microvessel density (MVD), an accepted histologic marker of tumor angiogenesis in several different human malignancies, including gastric cancer[30]. Recent years, the relationships between COX-2 expression, VEGF expression, tumor angiogenesis, and outcome parameters in gastric cancer patients have been reported[75-77]: COX-2 expression significantly correlates with VEGF, and the colocalization of these proteins is most frequent at the advancing edge of cancer cells[75]. MVD is higher in COX-2 and VEGF positive cases than in COX-2 and VEGF negative cases[76]. Co-expression of COX-2 and VEGF, age, lymph node status, and serosal invasion are

independent prognostic factors for overall survival in gastric cancer patients[77].

Interestingly, COX-1, the other isozyme expressed in tumor vascular endothelia, participates in tumor angiogenesis, because an anti-sense oligonucleotide of COX-1 suppresses *in vitro* angiogenesis induced by COX-2-overexpressing cells. Non-specific COX inhibitors reduce the growth and angiogenesis in cancer xenografts by inhibiting COX-1 in vascular endothelial cells, even when the tumor does not express COX-2. These results demonstrate that COX inhibitors suppress angiogenesis and tumor growth by inhibiting expression of angiogenic factors and vascular endothelial cell migration[79].

COX-2 expression and H pylori infection

H pylori infection is also a strong risk factor for the development of gastric cancer via the pathway of atrophy, intestinal metaplasia and dysplasia in chronic infection. Infection with *H pylori* up-regulates mediators of the inflammatory response, leading to the production of inflammatory cytokines and PGs which themselves may suppress cell-mediated immune responses and promote angiogenesis. These factors may also have effects on cell growth and survival signaling pathways, resulting in induction of cell proliferation and inhibition of apoptosis[81].

H pylori up-regulates COX-2 mRNA expression and PGE_2 synthesis in gastric cancer cells[82], and both pre-malignant and malignant gastric lesions demonstrate high COX-2 expression levels[83]. Therefore, *H pylori*-induced gastric carcinogenesis may be associated with the elevated expression of COX-2 in gastric epithelium. It is possible that NSAIDs may inhibit the replication and proliferation of *H pylori*[84,85], neutralize the increased COX-2 expression and PG synthesis and reverse the increased apoptosis and proliferation of epithelial cells associated with *H pylori* infection[86,89], thereby reducing the risk of gastric cancer[90,91]. Experimental models of cell proliferation have also shown that, in hyperplastic gastritis in the murine model infected with *H pylori*, treatment with etodolac could significantly decrease PGE_2 production and the thickness of gastric pits in the infected groups[87].

Whether eradication of *H pylori* can prevent gastric cancer remains contradictory. COX-2 expression remains in gastric epithelium even after successful eradication of *H pylori*[88]. This may suggest that combined treatment of COX-2 inhibitors and *H pylori* infection may be more useful in eliminating *H pylori*-induced COX-2 expression than either anti-*H pylori* antibiotics or COX-2 inhibitors alone. This issue deserves further study.

CONCLUSIONS AND FUTURE DIRECTIONS

Combining the evidence from humans, animal models and cell culture systems can establish the targeted inhibition of COX-2 was a viable approach to gastric cancer prevention and/or treatment. However, despite these successes, many questions remain unanswered such as the optimal dose and duration of treatment. Selective COX-2 inhibitors, which have an advantage of minimizing the gastrointestinal side-effects, have received wide attention. An exciting area for future investigation is to investigate whether COX-2 inhibitors can be used in the treatment of patients who have preneoplastic gastric lesions or suffer from gastric cancer, and whether combination therapy with COX-2 inhibitors and the drugs targeting other oncogenic pathways can lead to improved clinical outcomes.

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GASTRIC CANCER

Anticancer effect of Jinlongshe granules on *in situ*-transplanted human MKN-45 gastric cancer in nude mice and xenografted sarcoma 180 in Kunming mice and its mechanism

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Abstract

AIM: To study the antitumor effect of Chinese compound Jinlongshe (JLS) granules on sarcoma 180 and MKN-45 human gastric cancer cell lines *in vivo* and its mechanism.

METHODS: After establishment of S180 sarcoma (S180) and MKN-45 gastric cancer model of nude mice, the tumor-bearing mice were divided into 5 groups at random. Three experimental groups were respectively given the aqueous extract of JLS granules at doses of 120 g, 60 g and 20 g /(kg per 6/wk, *i.g*) for 3 wk in S180 and 6 wk in nude mice model. Positive control was given cyclophosphamide (Cy) at a dose of 50 mg/(kg per 3 /wk, *i.g*) for 3 wk in S180 models and 5- Fluorouracil (5-FU) 20 mg/(kg per 3 /wk, *i.g*) for 3 wk in nude mice model. Negative control was given normal saline (NS) at a dose of 0.18 g/(kg per 6/wk, *i.g*) respectively. After 3 wk in mice bearing S180 tumor and 6 wk in nude mice model, the experimental animals were sacrificed and the masses of tumor were weighed, and the rates of tumor inhibition of each treated group were calculated respectively. To determine the antitumor mechanisms, the morphological changes, cell cycle and apoptosis were observed in MKN-45 nude mice model. Annexin V-FITC/PI double staining FCM assay was used to further determine the live cells, apoptotic cells, necrotic cells and debris.

RESULTS: The inhibitory rates of JLS granules at the doses of 20 g/kg, 60 g/kg and 120 g/kg were 50.31%, 55.94% and 68.13% ($P < 0.01$) in nude mice models and 40.90%, 50.32% and 58.46% ($P < 0.01$) in S180 model. The inhibitory rate of Cy was 85.22% in S180 models and the inhibitory rate of 5-FU was 53.43% in nude mice model ($P < 0.01$). Nuclear chromatin and margination were observed under a transmission electron microscope (TEM). The G0/G1 phase was arrested, typical apoptotic peak appeared, the apoptotic rate was 22.81%-38.54% in three JLS granule-treated groups. Annexin V-FITC/PI double staining FCM assay showed that the apoptotic cells were 4.36%, 3.08%and 7.08% in three dosages, most cells were localized in the low right quadrant.

CONCLUSION: Jinlongshe granules possess anti-tumor effects on experimental tumor models *in vivo*, and apoptosis induction is one of its anti-tumor mechanisms.

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Key words: Jinlongshe granules; Sarcoma 180; Human MKN-45 gastric cancer lines; Traditional Chinese medicine

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INTRODUCTION

Gastric cancer is one of the most leading causes of cancer-related deaths worldwide[1]. Despite its decreasing trend in the Western world during the recent two decades, gastric cancer still accounts for about 23.2% of all cancer deaths in China[2-4]. Although surgery, chemotherapy, and irradiation are the mainstream therapeutic methods for gastric cancer, undesirable chemotherapy reaction and poor prognosis in the advanced gastric cancer patients are still the major problem for gastric cancer treatment[5]. Consequently, combination of multi-therapeutic methods may effectively improve gastric cancer patient's symptoms and prolong their survival time[6-7].

Jinlongshe (JLS) granules are an oral Chinese medicine compound, consisting of Chinese herbs (*Rhizome Arisaemat, Rhizoma Pinelliae, corium stomachium galli, Radix*

Glycyrrhizae preparata, etc.), developed by our department based on TCM theories of "phlegm" as the key factor in preventing and treating gastric cancer[8]. However, the exact antitumor mechanism of JLS granules is unclear. This study was to observe the anti-tumor effect of JLS granules and to determine its mechanism using sarcoma 180 and human gastric cancer cell line MKN-45 *in vivo*.

MATERIALS AND METHODS

Experimental animals

BALB/C nude mice (19-22 g) and Kunming mice (20-22 g) were provided by Experimental Animal Center of Chinese Academy of Sciences, Shanghai, China. All experimental animals were housed in specific-pathogen free (SPF) condition for 1 wk to adapt the surroundings before initiation of the experiment and had free access to food and water throughout the study.

Cancer cell lines

S180 cell line and MKN-45 human gastric denocarcinoma cell line were provided by Institute of Pharmaceutical Research, Shanghai, Chinese Academy of Sciences.

Drug samples and reagents

Crude JLS granules were purchased from Leiyunshang Company, Shanghai, China. An aqueous extract of JLS granules at a concentration of 6 g/mL of raw materials was provided by Shanghai Changzheng Hospital. The aqueous extract was diluted with purified water before use. Cyclophosphamide injection (Cy, Lot No. 040909) was produced in Hualian Pharmaceutical Co., Ltd, Shanghai, China. OB Biogel (Lot No.040514) was produced in Baiyun Medical Biogel Co., Ltd, Guangzhou, China. Ketamine hydrochloride injection (Lot No.02072332) was produced in Hengrui Pharmaceutical Co., Ltd, Jiangsu, China.

Establishment of in situ-transplanted human MKN-45 gastric carcinoma nude mouse model

The procedure of *in situ*-transplanted gastric cancer nude mouse model was referred to Xu and Chen Ling *et al*[9-10]. In brief, before transplantation, nude mice bearing gastric denocarcinoma MKN-45 cell lines were killed. Then the tumor was dissected and put into aseptical saline. Pieces of intact, fish meat-like tumor tissue of 1 mm × 1 mm × 1 mm in diameter from margin were prepared after removal of tumor capsule. Mice anesthetized with ketamine (50 mg/kg, ip) were sterilized with caseoiodine and a transverse incision was made on the left upper quadrant. After exposure of the greater curvature of stomach, serosa and muscular layer were incised with needle. Then tumor tissues were transplanted under serosa of stomach and one drop of OB biogel was applied to the tumor tissue. After 40 s, stomach was brought into abdominal cavity and abdominal wall was finally closed. The whole operation procedure was completed following the doctrine of aseptic manipulation.

Establishment of S180 model

This model was by subcutaneous injection as previously described[11]. Briefly, the S180 mouse sarcoma cells with ascites were harvested, diluted with sterilized saline at a ratio of 1:8 (cell concentration was adjusted to 1.0×10^6/mL), and inoculated subcutaneously into the right armpit region of Kunming mice.

Inhibitory effect of JLS granules on S180 and MKN-45 in vivo

Forty-eight hours after establishment of the MKN-45 gastric cancer model and 24 h after establishment of S180 model, the transplanted mice were divided into 5 groups according to the method described in "National Requirements for *in vivo* Screening of Anti-neoplastic Drugs"[12]. The dose of JLS granules was decided according to the clinical dose used in humans. The mice bearing S180 were killed by dislocation of cervical vertebra 3 wk after treatment. The nude mice bearing *in situ*-transplanted MKN-45 gastric cancer cell line were sacrificed 6 wk after treatment. The tumor weights were measured and the inhibitory rates were calculated according to the following equation: rate of inhibition (%) = (mean tumor wt of negative control -mean wt of treated group)/mean tumor wt of negative control ×100.

Morphologic assessment of apoptosis in MKN-45 nude mice

We selected NS group, 5-FU group and 60 g/kg JLS granules group for morphologic assessment of apoptosis in MKN-45 nude mice. Tumor tissue was cut into small pieces, homogenized into 1 mm × 2 mm, prefixed with 4% paraformaldehyde for 4 h and washed with PBS (0.1 mmol/L), post-fixed with 1% osmic acid, dehydrated in graded ethanol-acetonum, embedded in an Epon 812 mixture, and cut into sections on a ultramicrotome. After stained with uranyl acetate and lead citrate, the sections were examined under a Hitachi-800 transmission electron microscope.

Cell cycle and apoptosis in MKN-45 nude mice

Fresh tumor tissue separated from nude mice was chopped with mechanical method. Single cell-suspended solution was obtained with 200 filter screens. The suspensions were divided into two parts. One part was fixed with 70% ice-cold ethanol for at least 12 h at 4 ℃ and then washed with PBS again before examination. The fixed cells were treated with RNase A and stained with PI at 37 ℃ for 30 min. Analysis was performed with a Coulter FCM (EPICS-XL) and data were analyzed with Multicycle DNA content and cell analysis software.

Annexin V-FITC/PI double staining for FCM-assessed apoptosis

The Annexin V FITC kit (Jingmei Company, Lot No. 20050910) was used for detecting apoptosis according to manufacturer's instructions. The suspensions were washed twice and adjusted to a concentration of 1×10^6 cells/mL with ice cold 4 ℃ PBS. The Falcon tubes (12 mm × 75 mm, polystyrene round-bottom) were used in experiment, 100 μL of suspensions was added to each labeled tube, 10 μL of Annexin V-FITC and 10 μL 20 μg/mL PI were added into the labeled tube, incubated for at least 20 min at room temperature in the dark, then 400 μL of PBS

Table 1 Anti-tumor effect of JLS granules on mice bearing S180

Group	*n*	Tumor weight (g)	Inhibition rate (%)
Control	10	4.67 ± 3.27	-
120 g/kg	10	1.94 ± 2.03[ab]	58.46
60 g/kg	10	2.32 ± 2.83[ab]	50.32
20 g/kg	10	2.76 ± 2.32[ab]	40.90
Cy	10	0.69 ± 0.33[b]	85.22

[a]$P < 0.05$ *vs* Cy; [b]$P < 0.01$ *vs* control.

Table 2 Anti-tumor effect of JLS granules on MKN-45 cells in nude mice

Group	*n*	Tumor weight (g)	Inhibition rate (%)
Control	10	3.20 ± 0.96	-
120 g/kg	10	1.02 ± 0.57[b]	68.13
60 g/kg	10	1.41 ± 1.39[b]	55.94
20 g/kg	10	1.59 ± 1.12[b]	50.31
5-FU	8	1.49 ± 1.37[b]	53.43

[b]$P < 0.01$ *vs* control.

binding buffer was added to each tube without washing and analyzed by FCM as soon as possible (within 1 h).

Statistical analysis

Data were presented as mean ± SD and analyzed with SPSS 11.0 software. The analysis of variance (ANOVA) was used and least significant difference *t* test (LSD-*t*) was used for group comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Anti-tumor effect of JLS granules on S180

JLS granule at the doses of 20 g/kg, 60 g/kg and 120 g/kg significantly inhibited the growth of S180 subcutaneous tumor($P < 0.01$) in a dose-dependent manner, but lower than the inhibitory rates of Cy ($P < 0.05$) (Table 1).

Anti-tumor effect of JLS granules on in situ-transplanted gastric cancer MKN-45 cell lines

JLS granule at the doses of 20 g/kg, 60 g/kg and 120 g/kg significantly inhibited the growth of orthotopic-transplanted MKN-45 gastric cancer($P < 0.01$) in a dose-dependent manner, but no difference statistically compared with 5-FU ($P > 0.05$) (Table 2).

Morphology changes

Condensation of chromatin at margins of nuclei, disintegration of nucleolus, vacuoles in cytoplasm were observed in 60 g/kg group and 5-FU also had the same apoptotic appearance under TEM (Figure 1).

Assessment of cell cycle and apoptosis of MKN-45 cells in nude mice

The percentage of G0/G1 cells in JLS granules group was higher than that in NS group and 5-FU group in a dose-dependent manner ($P > 0.05$). The cell percentage of S phase in of JLS group and 5-FU group was lower than that in control group ($P < 0.05$), the apoptotic rate in 120 g/kg JLS group was higher than that in control group, 20 g/kg JLS group and 5-FU group ($P < 0.05$). The typical apoptotic peak was observed (Table 3, Figure 2).

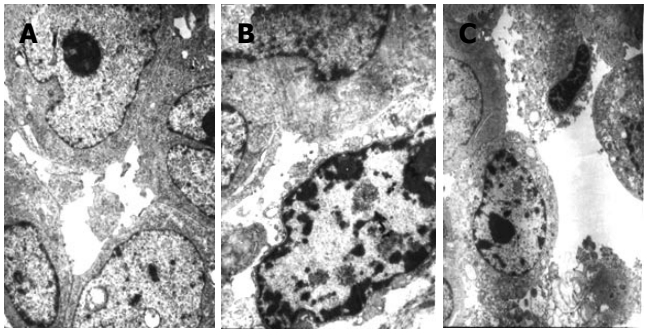

Figure 1 Ultra-structure observed under transmission electron microscopy in control group × 4 000 (**A**), 60 g/kg JLS group × 6 000 (**B**), 5-FU group × 3 000 (**C**).

Table 3 Effect of different treatments on cell cycle and apoptosis of MKN-45 cells (mean ± SD)

Group	*n*	G_0/G_1	G_2/M	S	Apoptotic rate
Control	10	42.40 ± 11.28	21.28 ± 11.43	36.92 ± 12.21	19.88 ± 14.83[c]
120 g/kg	10	50.58 ± 8.78	26.45 ± 12.53	14.56 ± 9.35[a]	38.54 ± 12.65[a]
60 g/kg	10	47.55 ± 7.02	26.51 ± 15.05	19.36 ± 12.69[a]	28.27 ± 11.25
20 g/kg	10	46.37 ± 12.09	25.47 ± 14.79	24.04 ± 13.27[a]	22.81 ± 10.65[c]
5-FU	8	41.78 ± 4.30	23.59 ± 11.97	19.97 ± 13.31[a]	24.97 ± 9.93[c]

[a]$P < 0.05$ *vs* control, [c]$P < 0.05$ *vs* 120 g/kg.

Assessment of apoptosis in MKN-45 nude mice

Results by Annexin V-FITC/PI double staining for FCM assay showed that the early apoptotic cells were 4.36%, 3.08% and 7.08% in the three JLS granules groups (Figure 3), and most of cell populations in 5-FU treated group were located in the up right quadrant and the cells on the lower right quadrant were not many as compared with JLS granules groups.

DISCUSSION

Tumorigenesis, in a sense, is due to the deregulation in cell proliferation and apoptosis[13-14], accordingly tumor treatment not only inhibits cell proliferation, but also induces apoptosis. More and more attention is paid to apoptosis in treatment of tumors and the effectiveness of anti-tumor agents is often evaluated[15,16]. Investigators have confirmed that many Chinese herbs have antitumor property and induction of apoptosis is one of the mechanisms[17-20]. But most of these studies aimed directly at single herbs, bioactive constituents or leading compounds extracted from herbs, and could not educe the multi-target action of Chinese compounds and traditional Chinese medicine (TCM) theories in disease therapy [21].

In TCM theories, "phlegm" is not only a pathologic metabolite, but also a causative agent. The metabolites such as ectopic hormone, growth factor and tumor

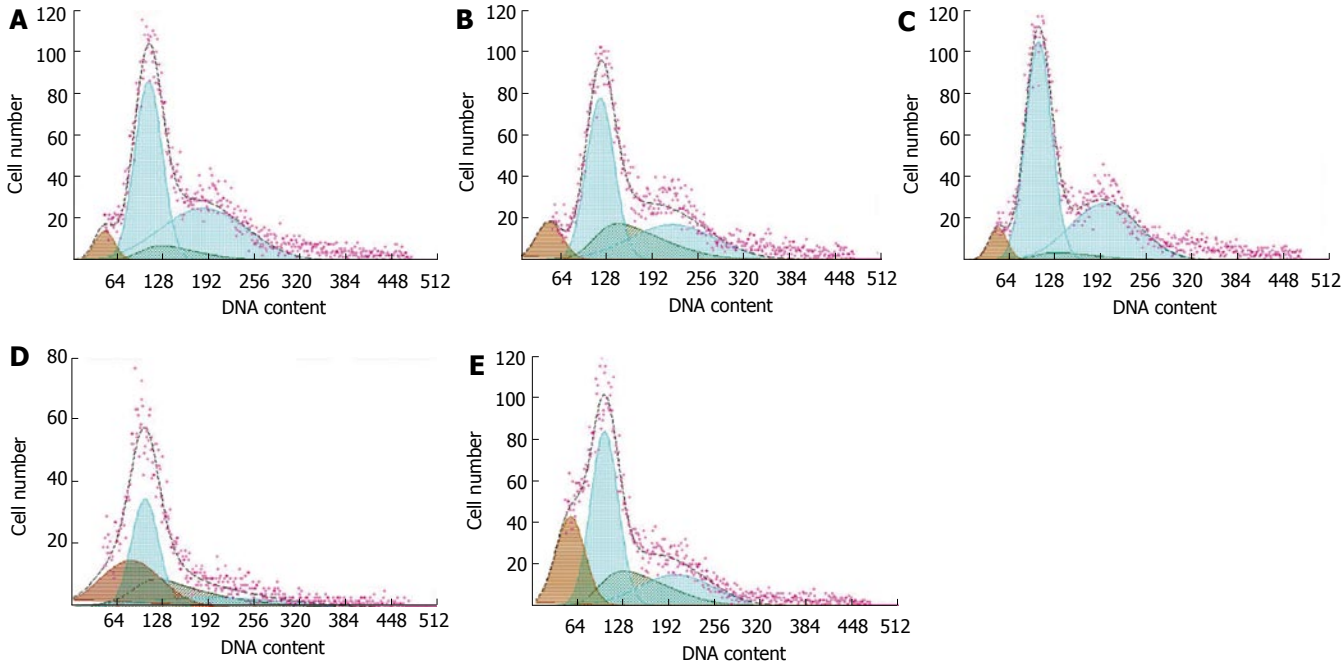

Figure 2 Histogram observed under FCM in 120 g/kg JLS group (**A**), 60 g/kg JLS group (**B**), 20 g/kg JLS group (**C**), control group (**D**), and 5-FU group (**E**).

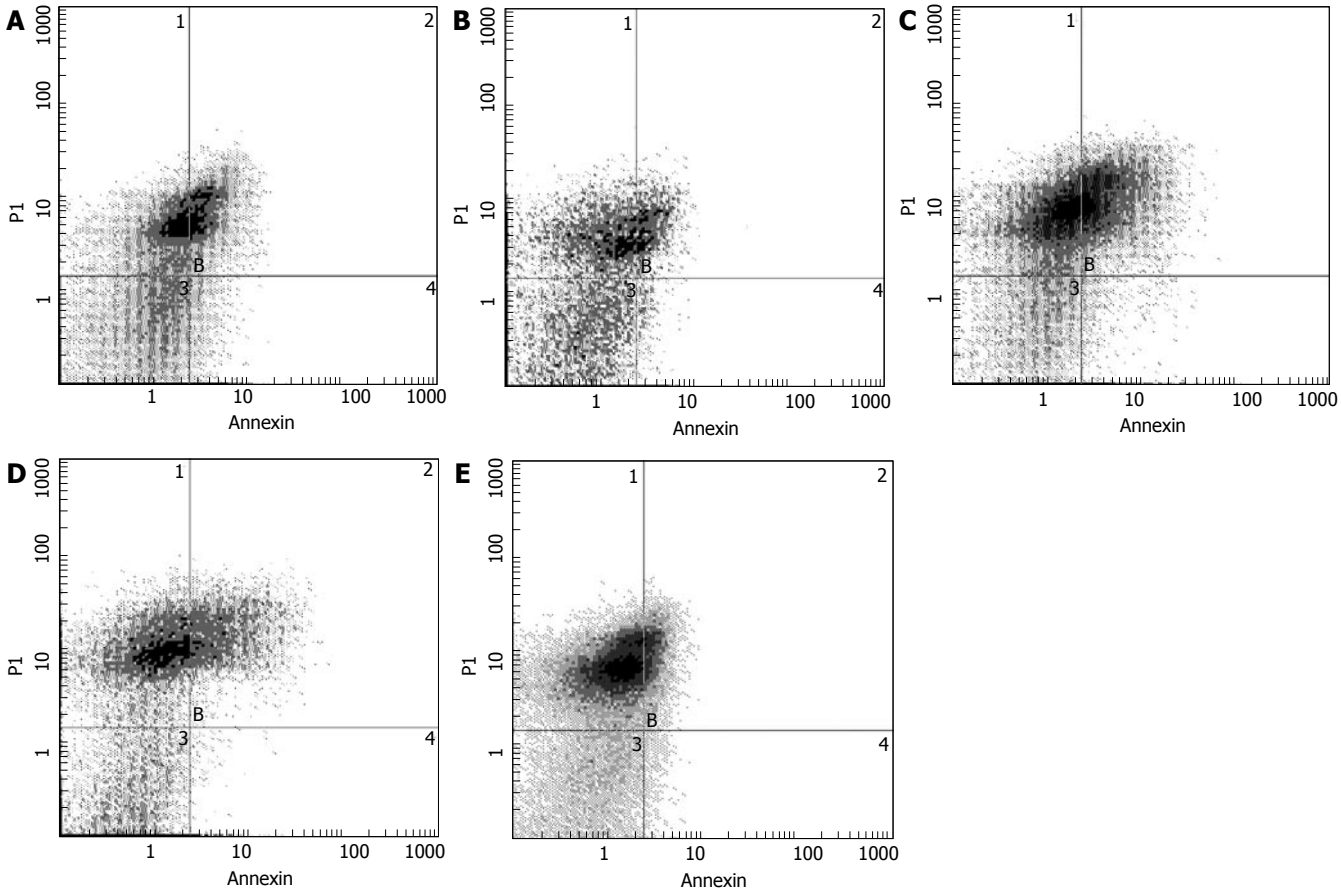

Figure 3 Cytogram observed by Annexin V-FITC/PI double staining assay in 120 g/kg JLS group (**A**), 60 g/kg JLS group (**B**), 20 g/kg JLS group (**C**), control group (**D**), and 5-FU group (**E**).

markers produced by tumor cells all belong to "phlegm", which can devitalize physiologic functions and stimulate tumor invasion and metastasis in turn. Therefore, "dispersing phlegm and eliminating stagnation" (cleaning metabolites produced by tumor) is one of the regimens for tumor treatment in TCM. JLS granules are an anti-

tumor compound prescription developed according to the methods of "dispersing phlegm and eliminating stagnation". Our previous experiments have shown that "dispersing phlegm and eliminating stagnation" inhibit could VEGF, Ras mRNA and protein expression[22-24] and have good antitumor and anti-metastasis potency *in vivo*. However whether it induces apoptosis is unclear. Our studies showed that JLS granules had anti-tumor effects on S180 and MKN-45 human gastric cancer. The ultra-structure of MKN-45 gastric cancer tissue treated with JLS granules showed that there were apoptosis changes. Cell cycle and apoptosis analysis also demonstrated that JLS granules could arrest MKN-45 cells at G0/G1 phase, decrease S phase cell populations. The typical apoptosis peak further confirmed its apoptosis-inducing effect.

In addition, changes in the plasma membrane are one of the earliest morphological features of apoptosis. At this time, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36kD Ca^{2+}-dependent phospholipid binding protein that has a high affinity for PS, thus Annexin V staining serves as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis[25-26]. Therefore, staining with Annexin V in conjunction with vital dyes such as propidiumiodide (PI) can identify early apoptotic cells (Annexin V positive, PI negative). In our study, cells treated with JLS granules were located in the low right quadrant and cells in 5-FU-treated group were located in the upper left quadrant, suggesting that the earlier apoptotic cells in JLS granules-treated groups are higher than those in 5-FU-treated group and that JLS granules induce apoptosis in human gastric cancer MKN-45 cells.

In conclusion, JLS granules have the inhibiting effects on human gastric carcinoma and induction of apoptosis may be one of its mechanisms.

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BASIC RESEARCH

Partial Beclin 1 silencing aggravates doxorubicin- and Fas-induced apoptosis in HepG2 cells

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Abstract

AIM: To investigate the role of Beclin 1 on the susceptibility of HepG2 cells to undergo apoptosis after anti-Fas antibody or doxorubicin treatment.

METHODS: Beclin 1 silencing was achieved using RNA interference. DNA ploidy, the percentage of apoptotic cells and the mitochondrial membrane potential were assessed by flow cytometry. Levels of Beclin 1, Bcl-X_L and cytochrome *c*, and the cleavage of poly (ADP-ribose) polymerase (PARP) were assayed by using Western blots.

RESULTS: Beclin 1 expression decreased by 75% 72 h after Beclin 1 siRNA transfection. Partial Beclin 1 silencing significantly increased the percentage of subG1 cells 24 and 40 h after treatment with doxorubicin or anti-Fas antibody, respectively, and this potentiation was abrogated by treatment with a pan-caspase inhibitor. Partial Beclin 1 silencing also increased PARP cleavage, mitochondrial membrane depolarization and cytosolic cytochrome *c*. The pro-apoptotic consequences of partial Beclin 1 silencing were not associated with a decline in Bcl-X_L expression.

CONCLUSION: Partial Beclin 1 silencing aggravates mitochondrial permeabilization and apoptosis in HepG2 cells treated with an anti-Fas antibody or with doxorubicin.

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Key words: Beclin 1; Apoptosis; HepG2 cells; Anti-Fas antibody; Doxorubicin

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INTRODUCTION

Beclin 1, a recently discovered tumor suppressor gene located on human chromosome 17q21, is mono-allelically deleted in 40%-70% of sporadic mammary, ovarian and prostate tumors[1]. Heterozygous beclin $1^{+/-}$ transgenic mice display a high incidence of spontaneous malignancies, including hepatocellular carcinoma (HCC), although the remaining beclin 1 allele is neither lost, mutated or silenced in these tumors[2,3]. These data have led to the conclusion that beclin 1 is a haploinsufficient tumor suppressor. One mechanism whereby beclin 1 haploinsufficiency can promote cancer is impaired autophagy, and increased cell proliferation[4]. However, it is not yet clear how Beclin 1 modulates cell death in cancer cells.

Several studies have reported a role of Beclin 1 in autophagic cell death. When embryonic fibroblasts from Bax/Bak double knockout mice were treated with etoposide, cells underwent a non-apoptotic cell death characterized by the presence of a number of autophagosomes/autolysosomes and the accumulation of both Beclin 1 and the APG5-APG12 complex[5]. Cell death was largely prevented by autophagy inhibitors as well as by silencing of Beclin 1 or APG5. In another study, treatment of mouse L929 fibroblastic cells or human U937 monocytoid cells with a pan-caspase inhibitor induced autophagic cell death[6]. Silencing of Beclin 1 or ATG7, another protein required for the formation of autophagic vacuoles, reduced z-VAD-induced cell death. Beclin 1 physically interacts with the anti-apoptotic proteins, Bcl-2 and Bcl-X_L[7], but the functional significance of this interaction on apoptosis is yet unclear. Thus, although beclin $1^{-/-}$ mouse embryos exhibited widespread cell death and died *in utero*, the *in vitro* apoptotic rate of beclin $1^{-/-}$ embryonic stem cells after UV irradiation or serum withdrawal was not significantly increased compared to wild-type cells[3]. By contrast, mice infected with a recombinant chimera of the neurotropic Sindbis virus combined with full-length human Beclin 1 exhibited

increased survival, fewer apoptotic brain cells and lower brain viral titers than mice infected with chimeras encoding a Beclin 1 protein lacking the Bcl-2 binding domain or with a premature stop codon[7]. However, these results did not provide information on whether the protective effects of Beclin 1 were a consequence of anti-apoptotic effects, anti-viral effects, or a combination of both mechanisms. Lastly, a recent study reported that the over-expression of Beclin 1 in MKN28 human gastric cancer cells stimulated *cis*-diamminedichloroplatinum (CDD)- or doxorubicin-induced cell death, while a Beclin 1 siRNA protected the cells from apoptosis in the same experimental conditions[8].

We have previously observed a significant correlation between Beclin 1 and Bcl-X_L mRNA expression in human hepatocarcinoma (unpublished results), suggesting that Beclin 1 over-expression may prevent hepatocyte apoptosis. In order to test this assumption, we investigated the effect of Beclin 1 silencing on the response of HepG2 cells to apoptotic stimuli. In the present study, we showed that partial Beclin 1 silencing aggravates the mitochondrial permeabilization and the apoptotic response induced by an agonistic anti-Fas antibody or by doxorubicin in a human HCC cell line.

MATERIALS AND METHODS

Materials

HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mL/L fetal calf serum and 1% penicillin/streptomycin sulfate (Gibco, Invitrogen).

Small interfering RNA (siRNA) duplexes were purchased from Dharmacon (Lafayette, CO). We used the beclin 1 siRNA: 5'-CAGUUACAGAUGGAGCUAA tt-3' (nt 655-673), which is located downstream from the initiation codon of the human beclin coding region, and a luciferase siRNA as a control siRNA: 5'-CUUACGCU GAGUACUUCGAtt3'. HepG2 cells (15-25 × 10^6 cells) were collected by trypsinization. The pellet was washed with serum-free OptiMEM (Gibco, Invitrogen) without antibiotics, and cells were resuspended in 500-600 μL of the same medium containing 12 μmol/L siRNA. After 10 min on ice, cells were electroporated at 230 V, 960 μF. Cells were resuspended in DMEM containing 100 mL/L fetal calf serum without antibiotics, and were plated in 60 mm Petri dishes (approximately 10^6 cells per dish). Antibiotics were added 3 h later.

In a first treatment protocol, 5 μg/mL of an agonistic anti-Fas antibody (7C11, Immunotech, Beckman Coulter, Miami, FL) was added 24 h after siRNA transfection, with or without 10 μmol/L benzyloxycarbonyl-valyl-alanyl-aspartic acid (o-methyl)-fluoro-methylketone (z-VAD-fmk) (Alexis, Coger, Paris, France), a pan-caspase inhibitor, and apoptosis was detected 40 h later, except for the assessment of cytochrome *c* release, which was detected at 24 h.

In a second treatment protocol, 6 μmol/L doxorubicin (Sigma, Saint-Louis, MO) was added 48 h after siRNA transfection, with or without 100 μmol/L z-VAD-fmk, and apoptosis was detected 24 h later except for the assessment of cytochrome c release, which was detected at 20 h.

Measurement of Beclin 1 mRNA expression by ribonuclease protection assay (RPA)

Attached HepG2 cells were washed twice with PBS and lysed in 5 mol/L guanidine thiocyanate and 0.1 mol/L EDTA (pH 7.4). RPA was carried out on cell lysates without previous RNA extraction[9]. The following DNA fragments were used to prepare riboprobes: a 200-bp fragment (nt 554-753, accession no. AF139131) from human beclin 1, and a 131-bp fragment (nt 553-683, accession no. NM00101) from human S6 used to normalize mRNA levels. The RPA and the separation of the protected mRNA fragments by polyacrylamide gel electrophoresis were performed as previously described[9]. Gel radioactivity was assessed with an Instant Imager (PerkinElmer, Courtaboeuf, France), and the ratio of the studied mRNA to the S6 mRNA was computed.

Western blot analysis of Beclin 1 and Bcl-X_L

Attached HepG2 cells were washed twice with PBS, harvested in PBS, and pelleted by centrifugation. The pellet was weighed and suspended in 2 × Laemmli buffer. Proteins were separated on SDS-polyacrylamide gels (70 g/L polyacrylamide for Beclin 1 and 120 g/L for Bcl-X_L) and transferred onto nitrocellulose membranes. After blocking, membranes were incubated for 1 h with either the anti-Beclin 1 antibody supplied by Dr Yoshimori[10], or an anti-Bcl-$X_{S/L}$ antibody (S-18) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Immunotech, Beckman Coulter). Specific bands were detected by an enhanced chemiluminescence system (Pierce, Rockford, IL). Membranes were subsequently probed with a monoclonal anti-β-actin antibody (Sigma), and the β-actin signal was used to normalize protein loading.

Poly(ADP-ribose) polymerase (PARP) cleavage

Attached and floating HepG2 cells were harvested in culture medium and pelleted by centrifugation. Nuclear proteins were extracted according to Sadowski and Gilman[11]. After protein measurement (Biorad Reagent, Pierce), nuclear extracts underwent SDS-7% polyacrylamide gel electrophoresis and blotting as described above, and were revealed with a monoclonal anti-human PARP antibody (clone C2-10, BD Pharmingen, San Diego, CA).

Assessment of cell DNA content by flow cytometry

Pooled, free and adherent HepG2 cells were centrifuged, washed with PBS and fixed with 700 mL/L cold ethanol. The pellet was resuspended in 100 μL RNAse A (180 μg/mL) and incubated at room temperature for 30 min. Propidium iodide (Merck, Darmstadt, Germany; final concentration, 50 μg/mL) was added, and cells were incubated at room temperature in the dark for 15 min. The percentage of cells with a low DNA content (subG1 cells) was quantified by flow cytometry using an Epics Elite ESP coulter cytometer (Beckman coulter, Fullerton, CA). Propidium iodide was excited at 488 nm and fluorescence was analyzed at 630 nm.

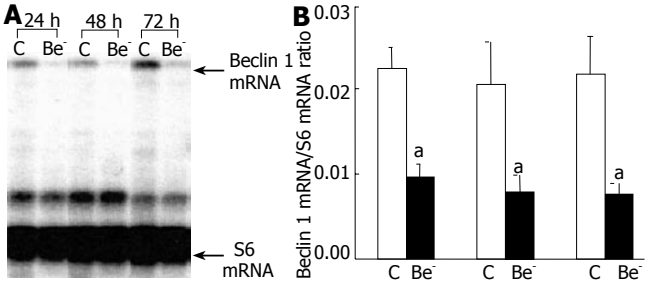

Figure 1 Beclin 1 mRNA expression after transfection of HepG2 cells with a Beclin 1 siRNA or a control siRNA. **A:** Representative RPA gel; **B:** Quantification of the Beclin1/S6 mRNA ratio. Results are means ± SE for 4 experiments ($^aP < 0.05$ *vs* C).

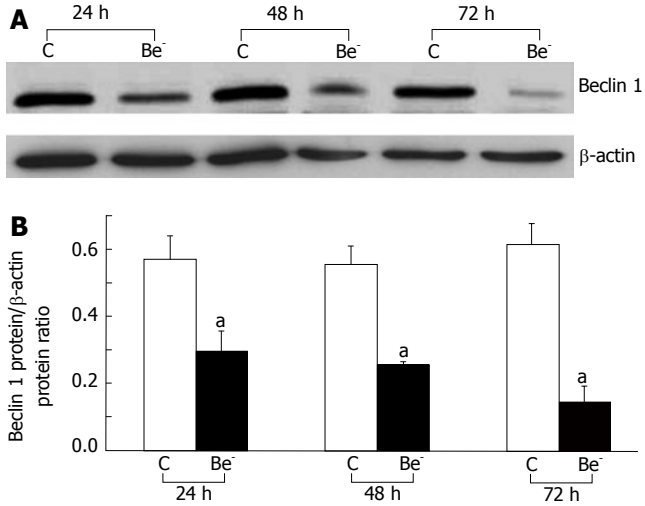

Figure 2 Beclin 1 protein expression after transfection of HepG2 cells with a Beclin 1 siRNA or a control siRNA. **A:** Representative Western blot of Beclin 1 and β-actin; **B:** Quantification of the Beclin 1 protein normalized to β-actin. Data are means ± SE for three experiments ($^aP < 0.05$ *vs* C).

Assessment of the mitochondrial membrane potential by flow cytometry

To determine the mitochondrial membrane potential, we used DIOC6(3) (Molecular Probes, Eugene, OR), a lipophilic cationic dye, which accumulates into polarized mitochondria. Free and adherent HepG2 cells were collected, rinsed with PBS, and suspended in the culture medium. DIOC6(3) was added at a final concentration of 100 nmol/L, and cell suspensions were incubated at 37 ℃ in the dark for 30 min. Propidium iodide (1.5 μmol/L) was added before flow cytometry to exclude dead cells and to only take into account the fluorescence DIOC6(3) in living cells. DIOC6(3) fluorescence was measured by flow cytometry. DIOC6(3) was excited at 488 nm and detected at 510 nm.

Measurement of cytochrome c release

Mitochondrial and cytosolic extracts were prepared using the ApoAlert® cell fractionation kit (BD Biosciences Clontech, Mountain View, CA, USA). Equal levels of protein were separated on SDS-12.5% polyacrylamide gel electrophoresis, transferred and immunoblotted with an anti-cytochrome *c* antibody supplied with the kit.

Statistical analysis

Data were expressed as means ± SE. An unpaired Student's *t* test was used to assess statistical differences between HepG2 cells transfected with the control siRNA or the Beclin 1 siRNA. A paired Student's *t* test was used to assess differences resulting from treatments in control siRNA- or in Beclin 1 siRNA-transfected HepG2 cells.

RESULTS

Effects of partial Beclin 1 silencing on Fas- and doxorubicin-mediated apoptosis

We used RNA interference to partially silence the Beclin 1 gene in HepG2 cells. The Beclin 1 mRNA was decreased by 57%, 61% and 65%, 24, 48 and 72 h after transfection of the Beclin 1 siRNA, respectively (Figure 1), and the Beclin 1 protein was decreased by 48%, 54% and 76%, respectively (Figure 2).

We then measured the percentage of cells with a decreased DNA content (subG1 cells) as a consequence of DNA fragmentation after treatment of transfected cells with doxorubicin or with an agonistic anti-Fas antibody, which both trigger apoptotic cell death in HepG2 cells[12,13]. We compared the percentage of cells with a subG1 DNA content in HepG2 cells transfected with the control siRNA (control cells) or the Beclin 1 siRNA (Be^- cells) (Figure 3). The percentage of subG1 cells was not significantly increased in the untreated Be^- cells compared to the untreated control cells (Figure 3B). However, after exposure to the anti-Fas antibody or doxorubicin, the increase in subG1 cells was much higher in the Be^- cells compared to the control cells (Figure 3B). In both control cells and Be^- cells, the pan-caspase inhibitor z-VAD-fmk markedly decreased subG1 cells after treatment with the anti-Fas antibody or doxorubicin (Figure 3C).

We also studied the apoptotic response of HepG2 cells by assessing PARP cleavage (Figure 4). The cleaved p85 fragment of PARP was not detected in the untreated control cells or untreated Be^- cells. After treatment with the anti-Fas antibody, the cleaved p85 fragment was still absent in the treated control cells, but present in the treated Be^- cells. After treatment with doxorubicin, the p85 fragment was more abundant, and the uncleaved p116 fragment less abundant, in the Be^- cells compared to the control cells (Figure 4).

Since the apoptotic effects of Fas or doxorubicin are amplified by mitochondria[14-16], we determined whether Beclin 1 depletion increases the mitochondrial pathway of apoptosis by first assessing changes in the mitochondrial membrane potential (Figure 5). The percentage of living cells with a low mitochondrial membrane potential was not significantly increased in the untreated Be^- cells compared to the untreated control cells. After treatment with the anti-Fas antibody or doxorubicin, the percentage of living cells with a low mitochondrial membrane potential was significantly higher in the Be^- cells than that in the control cells (Figure 5). We next investigated whether Beclin 1 silencing increases the release of mitochondrial cytochrome *c* into the cytosol after treatment with the anti-Fas antibody or doxorubicin[14,16]. We firstly checked that the cytosolic fraction was not contaminated by

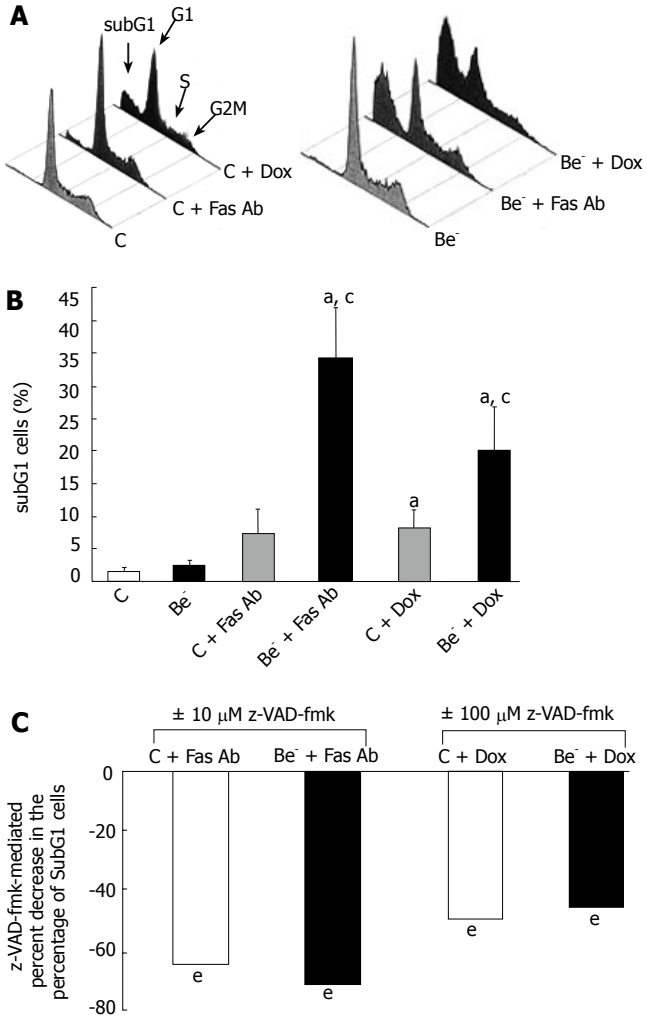

Figure 3 Percentage of subG1 cells after treatment of siRNA-transfected HepG2 cells with an agonistic anti-Fas antibody or doxorubicin. **A:** Representative flow cytometry analysis of the cell cycle; **B:** Quantification of the subG1 peak. Results are means ± SE for 5 to 8 experiments ($^aP < 0.05$ *vs* untreated cells; $^cP < 0.05$ *vs* cells transfected with the control siRNA); **C:** Effects of z-VAD-fmk. The figure shows the mean percent decrease in subG1 cells for 3 to 5 experiments ($^eP < 0.05$ between cells incubated with and without z-VAD-fmk).

mitochondria by assessing COX4 (data not shown). Cytosolic cytochrome *c* was increased by the anti-Fas antibody or the doxorubicin treatment in the control cells, and this increase was further enhanced in the Be⁻ cells compared to the control cells, particularly after treatment with the anti-Fas antibody (Figure 6).

To test whether this effect could be mediated by decreased Bcl-X_L expression, we assessed this protein in the untreated and treated cells (Figure 7). Bcl-X_L was not significantly decreased 24, 48 or 72 h post-transfection in the cells transfected with Beclin 1 siRNA compared to the cells transfected with the control siRNA (Figures 7A-B). Treatment with the anti-Fas antibody caused no further changes (Figures 7C-D). Although treatment with doxorubicin tended to decrease Bcl-X_L expression in the control cells, and even more in the Be⁻ cells, the doxorubicin-mediated decline in Bcl-X_L did not differ significantly between the control cells and Be⁻ cells (Figures 7C -D).

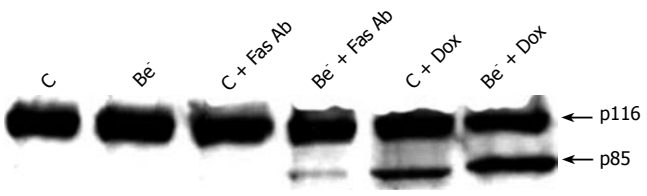

Figure 4 Western blot analysis of PARP cleavage after treatment of siRNA-transfected HepG2 cells with an agonistic anti-Fas antibody or doxorubicin.

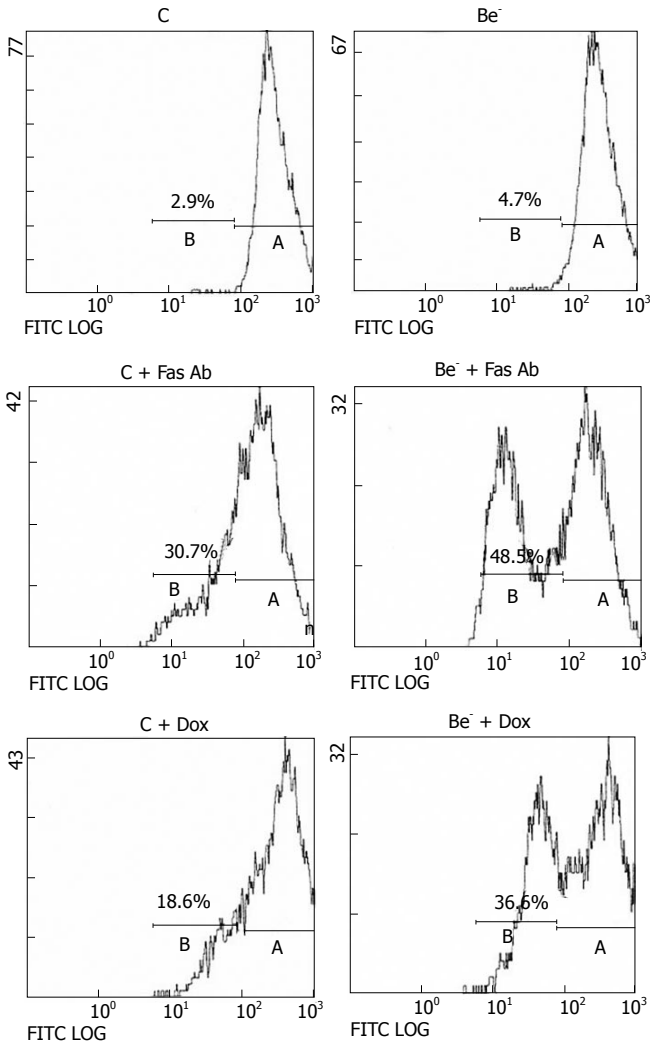

Figure 5 Assessment of the mitochondrial membrane potential in HepG2 living cells after siRNA transfection and treatment with an agonistic anti-Fas antibody or doxorubicin (Dox). In four to five different experiments, the percentage (mean ± SE) of cells with a low mitochondrial membrane potential was 4.5 ± 1.3% in C cells, 7.7 ± 2.9% in the Be- cells, 29.1 ± 4.9% in C cells treated with the Fas Ab, 52.5 ± 7.1% in Be- cells treated with the Fas Ab ($P < 0.05$ *vs* treated C cells), 23.6 ± 3% in C cells treated with Dox, and 38.8 ± 3.8% in Be- cells treated with Dox ($P < 0.05$ *vs* treated C cells).

DISCUSSION

Although beclin 1 haploinsufficiency is involved in several extrahepatic human cancers[1,4], and increases the frequency of various tumors, including HCC in transgenic mice[2,3], its role in human HCC is still not clear[17], and its potential involvement in liver apoptosis has not been investigated

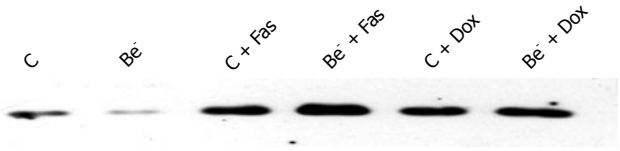

Figure 6 Assessment of cytochrome c release in HepG2 cells after siRNA transfection and treatment with an agonistic anti-Fas antibody or doxorubicin.

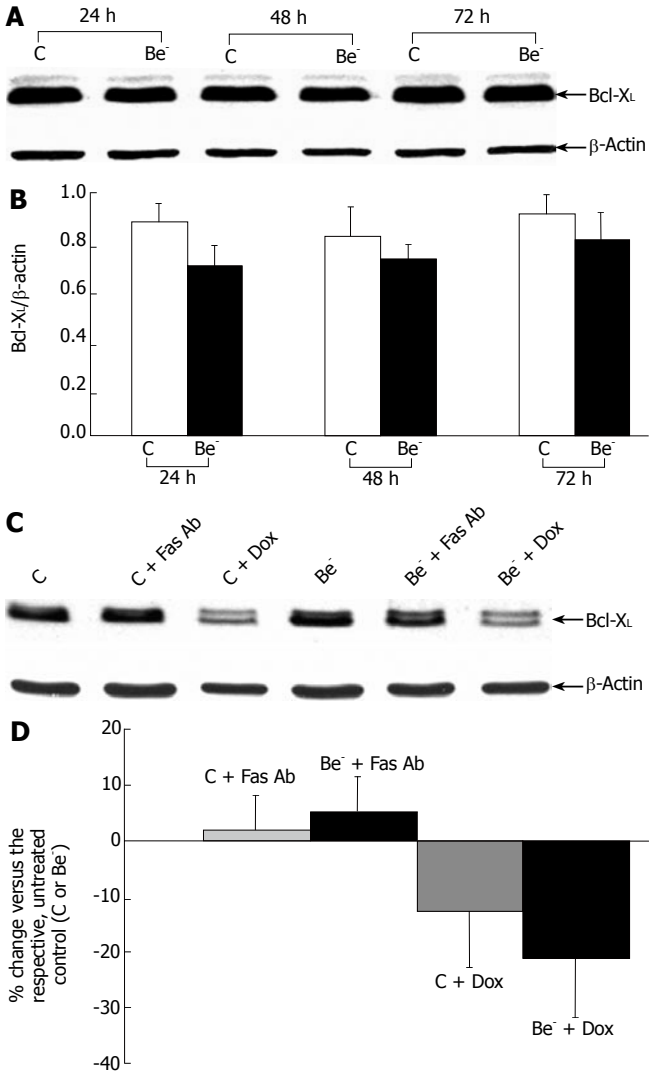

Figure 7 Expression of the Bcl-X_L protein after siRNA transfection, and treatment with an agonistic anti-Fas antibody or doxorubicin (Dox). **A:** Representative Western blot of Bcl-X_L and β-actin; **B:** Quantification of the Bcl-X_L/β-actin ratio (mean ± SE for 5 experiments); **C:** Representative Western blot of Bcl-X_L and β-actin after incubation with or without Dox or an anti-Fas; **D:** Quantification of the Fas Ab- or Dox-induced change in Bcl-X_L expression (means ± SE for 4 to 5 experiments).

yet. The aim of the present study was to assess the effects of partial Beclin 1 silencing on the susceptibility of HepG2 human hepatoma cells to undergo apoptosis.

In the cells transfected with a Beclin 1 siRNA (Be^- cells), the Beclin 1 protein was decreased by 50% at 24 h and by 75% at 72 h compared to the cells transfected with a control siRNA (control cells). Although the spontaneous apoptotic rate of the Be^- cells was not significantly increased over that of the control cells, their apoptotic response to doxorubicin or an agonistic anti-Fas antibody was markedly aggravated (Figure 3). The percentage of subG1 cells was much higher in the treated Be^- cells compared to the treated control cells (Figures 3A-B) and dropped significantly when cells were pre-treated with the pan-caspase inhibitor, z-VAD-fmk (Figure 3C). In addition, PARP cleavage was more extensive in the treated Be^- cells than that in the treated control cells, especially after doxorubicin poisoning (Figure 4).

Two reasons prompted us to investigate the potential effect of Beclin 1 depletion on the mitochondrial pathway. Firstly, Beclin 1 physically interacts with Bcl-2 or Bcl-X_L[7,8], two anti-apoptotic proteins predominantly localized to the mitochondrial membrane that prevent loss of mitochondrial membrane potential and cytochrome *c* release[18-20]. Secondly, the apoptotic effects of Fas or doxorubicin are amplified by mitochondria, in particular in hepatocytes and hepatoma cell lines[14-16]. The dissipation of mitochondrial membrane potential after anti-Fas antibody or doxorubicin treatments was more severe, and the cytochrome *c* release was more abundant in the Be^- cells than that in the control cells (Figures 5 and 6), suggesting that Beclin 1 depletion activates the mitochondrial pathway of apoptosis. In a previous study, the over-expression of full-length human Beclin 1 was associated with decreased neuronal apoptosis in mice infected with the neurotropic Sinbis virus, whereas a deleted Beclin 1 construct lacking the region interacting with Bcl-2 or Bcl-X_L was not protective[7]. This previous observation may suggest that the interaction of Beclin 1 with either Bcl-2 or Bcl-X_L is essential for the anti-apoptotic effects of Beclin 1[7]. Since several studies have reported that Bcl-2 is not constitutively expressed in HepG2 cells[21-23] and Bcl-2 over-expression does not protect these cells from Fas or doxorubicin treatment[22-24], Bcl-X_L seems to be the major anti-apoptotic protein in the HepG2 cell line. In the present study, partial Beclin 1 silencing increased apoptosis without significantly decreasing the Bcl-X_L protein (Figure 7), suggesting that normal Beclin 1 levels are required to protect HepG2 cells from apoptotic stimuli, possibly by maintaining the expression of the Bcl-X_L-Beclin 1 complex. How does the interaction between Beclin 1 and Bcl-X_L or other proteins act to modulate mitochondrial permeabilization and apoptosis remains to be determined.

In contrast to our results, Furuya *et al*[8] instead observed that Beclin 1 over-expression aggravated CDD- or doxorubicin-induced apoptosis in a gastric cancer cell line, while Beclin 1 silencing had the opposite effects[8]. However, Beclin 1 silencing did not protect another gastric cancer cell line (with lower Bcl-2 and Bcl-X_L expression) against CDD-mediated apoptosis. It thus appears that Beclin 1 differently modulates apoptosis in different cell lines. Although reasons for these differences are unknown, changes in the molecular environment of Beclin 1 from one cancer cell line to another might be involved, including different expression of Bcl-2 and Bcl-X_L[8], and other pro-apoptotic proteins of the Bcl-2 family interacting with these proteins, but not with Beclin 1[7,8,24,25]

In conclusion, our data demonstrate that partial Beclin 1 silencing augments the mitochondrial permeabilization and the apoptosis induced by Fas stimulation or doxorubicin treatment in hepatoma-derived HepG2 cells. As Takehara *et al*[21] reported that down-expression of Bcl-XL by antisense oligonucleotide aggravates apoptosis induced by staurosporine or serum deprivation in HepG2 cells, the use of both Beclin 1 and Bcl-X_L antisense oligonucleotides in combination with chemotherapeutic drugs might efficiently trigger apoptosis in human hepatocarcinoma. The different effects of Beclin 1 on apoptosis in different cell lines[3,7,8] show that Beclin 1, unlike Bcl-2 or Bcl-X_L, can be pro-apoptotic, anti-apoptotic or non-effective, depending on the cellular context.

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BASIC RESEARCH

Down-regulation of survivin expression by small interfering RNA induces pancreatic cancer cell apoptosis and enhances its radiosensitivity

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Abstract

AIM: To investigate the inhibitory effect of small interfering RNA (siRNA) on the expression of survivin in pancreatic cancer cell line PC-2 and the role of siRNA in inducing PC-2 cell apoptosis and enhancing its radiosensitivity.

METHODS: A siRNA plasmid expression vector against survivin was constructed and transfected into PC-2 cells with Lipofectamine™ 2000. The down regulation of survivin expression was detected by semi-quantitive RT-PCR and immunohistochemical SP method and the role of siRNA in inducing PC-2 cell apoptosis and enhancing its radiosensitivity was detected by flow cytometry.

RESULTS: The sequence-specific siRNA efficiently and specifically down-regulated the expression of survivin at both mRNA and protein levels. The expression inhibition ratio was 81.25% at mRNA level detected by semi-quantitive RT-PCR and 74.24% at protein level detected by immunohistochemical method. Forty-eight hours after transfection,apoptosis was induced in 7.03% cells by siRNA and in 14.58% cells by siRNA combined with radiation.

CONCLUSION: The siRNA plasmid expression vector against survivin can inhibit the expression of survivin in PC-2 cells efficiently and specifically. Inhibiting the expression of survivin can induce apoptosis of PC-2 cells and enhance its radiosensitivity significantly. RNAi against survivin is of potential value in gene therapy of pancreatic cancer.

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Key words: RNA interference; Survivin; Pancreatic cancer; Radiation

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INTRODUCTION

Survivin, a structurally unique member of the inhibitors of apoptosis protein (IAP)family, is involved in the control of cell division and inhibition of apoptosis. Survivin inhibits apoptosis via its baculovirus inhibitor of apoptosis repeat (BIR) protein domain by either directly or indirectly interfering with the function of caspases. Survivin is also a chromosomal passenger protein that is required for cell division. Survivin is expressed in embryonic tissues as well as in the majority of human cancers, but not in most normal adult tissues. The cancer-specific expression of survivin, coupled with its importance in inhibiting cell apoptosis and regulating cell division, makes it a useful diagnostic marker and a potential target for cancer treatment[1].

RNA interference (RNAi) is a process of post transcriptional gene silencing (PTGS) in which double-stranded RNA (dsRNA) inhibits gene expression in a sequence-dependent manner *via* degradation of the corresponding mRNA [2-5]. RNAi is initiated by an event while dsRNAs are recognized by Dicer, a member of the RNase III protein family[6]. The Dicer enzyme cleaves dsRNAs into 21 to 23 nucleotide short siRNAs. These siRNA duplexes are incorporated into a protein complex called the RNA-induced silencing complex, which recognizes and cleaves the cognate mRNA[7, 8]. Recently, Elbashir and his colleagues reported that introduction of synthetic 21nt siRNA into

mammalian cells could suppress gene expression in a sequence-specific manner[9]. By using dsRNAs of such a small size, the activation of dsRNA-dependent protein kinase and 2', 5'- oligoadenylate synthetase, which occurs as an interferon response to long dsRNA (i.e. longer than 30nt), leading to a global decrease in protein synthesis, is avoided [10,11]. Thus, siRNA has now become a powerful tool for studies on gene function, cancer and viral disease therapy. In this study, a siRNA against survivin plasmid expression vector was constructed and transfected into PC-2 cells. The changes of survivin expression following RNAi and the role of siRNA in inducing PC-2 cell apoptosis and enhancing its radiosensitivity were investigated, which may lay a foundation for further studies on the functions of survivin and genetic therapy involved in human pancreatic cancer.

MATERIALS AND METHODS

Cell line and culture

Human pancreatic cancer cell line PC-2 was purchased from the Medical Experimental Animal Center of the Fourth Military Medical University. The cells were cultured in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum (FBS), 1×10^5 U/L penicillin and 0.1 g/L streptomycin in a humidified incubator containing 50 mL/L CO_2 at 37 ℃.

Construction of siRNA plasmid expression vector against survivin

Plasmid vector Pgenesil-1 was purchased from Wuhan Genesil Biotechnology Co, Ltd. Aiming at the sequence of survivin[12] (5'-GGA CCA CCG CAT CTC TAC A-3'), two DNA chains were synthesized as following: 5'-GATCC GGA CCA CCG CAT CTC TAC A TTCAAGACG TGT AGA GAT GCG GTG GTC C TTTTTT GAATTC A-3' and 3'-G CCT GGT GGC GTA GAG ATG T AAGTTCTGC ACA TCT CTA CGC CAC CAG G AAAAAA CTTAAG TTCGA-5'. The structure of the DNA chains is *Bam*HI + sense chain + loop + antisense chain + termination signal + *Eco*RI + *Hin*dIII. Equal amount of the two DNA chains was annealed and phosphorylated, then the double-stranded DNA was ligated with the linearized plasmid vector Pgenesil-1 completely digested with *Bam*HI and *Hin*dIII. The ligated plasmid DNA was transfected into competent cells $DH_{5\alpha}$ which were then seeded on solid LB medium containing 0.05 g/L kanamycin and cultured at 37 ℃ overnight. Three monoclonal colonies were picked out and seeded in 3 mL LB culture fluid containing 0.05 g/L kanamycin and cultured at 37 ℃ overnight in a rocking bed. The plasmid was extracted and verificated by digesting with restriction endonucleases and sequencing. The new plasmid was named Pgenesil-sur(+). The negative control plasmid Pgenesil-sur(-) was constructed as the same process. The target sequence is 5'-GAC TTC ATA AGG CGC ATG C-3' and does not have any homology with human beings or mice. The sequences of the two DNA chains are 5'-GATCC GAC TTC ATA AGG CGC ATG C TTCAAGACG GCA TGC GCC TTA TGA AGT C TTTTTT GTCGAC A-3' and 3'-G CTG AAG TAT TCC GCG TAC G AAGTTCTGC CGT ACG CGG AAT ACT TCA G AAAAAA CAGCTG TTCGA -5'. Their structure is *Bam*HI + sense chain + loop + antisense chain + termination signal + *Sal*I + *Hin*d III.

Extraction of plasmids

The plasmid extraction kit without endotoxin was purchased from Beijing Tianwei Corporation. The plasmids were extracted following the manufacturer's instructions. The concentration and purity of the plasmids were detected with an ultraviolet spectrophotometer. The plasmids were stored at -20 ℃ for following experiments.

Transfection of plasmids

Transfection reagent Lipofectamine™2000 was purchased from Invitrogen Corporation. Transfection was performed following the manufacturer's instructions. The ratio of plasmids: Lipofectamine™2000 was 1:3. Twenty-four hours before the transfection, the common complete medium was replaced by the antibiotics- free medium containing serum. Six hours after the transfection, the medium was replaced by the common complete medium again. Twelve hours after the transfection, expression of EGFP in PC-2 cell was observed under an inverse fluorescence microscope.

Expression of survivin mRNA detected by semi-quantitive RT-PCR

PC-2 cells were seeded in 6cm culture capsules and divided into blank control group, negative control group and positive experiment group. Each group contained 3 culture capsules. Only Lipofectamine™2000 was used for the transfection in the blank control group, plasmid Pgenesil-sur(-) was used for the transfection in the negative control group and plasmid Pgenesil-sur(+) was used for the transfection in the positive experiment group. Twenty-four hours after the transfection, 1×10^6 cells were collected and total RNA was extracted using the Trizol reagent following the manufacturer's instructions. The concentration and purity of the total RNA were detected with ultraviolet spectrophotometer. RT-PCR was performed by two-step method. Synthesis of cDNA was performed using the RevertAid™ first chain cDNA synthesis kit following the manufacturer's instructions. PCR was performed using the one tube convenient PCR kit purchased from Beijing Tianwei Corporation. Amplification of human GAPDH served as an internal standard. The primers used are 5' -CGA AGT CAA CGG ATT TGG TCG TAT-3' (forward primer) and 5'-AGC CTT CTC GGT GGT GAA GAC-3' (reverse primer). The amplification product was 306bp.The primers of survivin are 5'-GCA TGG GTG CCC CGA CGT TG-3' (forward primer) and 5'-GCT CCG GCC AGA GGC CTC AA -3' (reverse primer). The amplification product was 447bp. The PCR products were separated in 1.5% agarose gels, visualized by staining with ethidium bromide. Semi-quantitative analysis was performed with the Dolphin 1D software. The expression intensity of survivin was denoted with the ratio of the photodensity of the RT-PCR products of survivin and GAPDH. The inhibition ratio of survivin expression was calculated with the following formula: inhibition ratio of survivin expres-

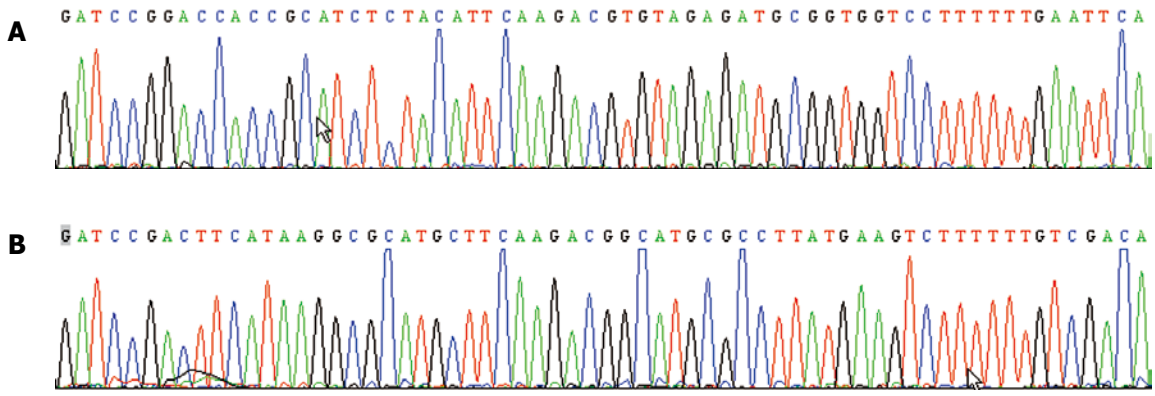

Figure 1 Reports of the sequencing of plasmid Pgenesil-sur(+) (**A**) and plasmid Pgenesil-sur (-) (**B**).

sion = (1-the expression intensity of survivin in the observation group/ the expression intensity of survivin in the blank control group)×100%.

Expression of survivin protein detected by immunohistochemical method

PC-2 cells were seeded in 24-well plates with a small square cover slip in each well. Grouping and transfection were performed the same as above. Each group contained 8 wells. Twenty-four hours after the transfection, the cells were fixed with cold 65% liquor pyroaceticus. The expression of survivin protein was detected by immunohistochemical SP method. The anti-survivin antibody was a rabbit anti-human monoclonal antibody purchased from Beijing Zhongshan Corporation. The working concentration was 1:50. A petroline section of breast cancer expressing survivn positively was used as a positive control, PBS was used to replace the anti-survivn antibody to make a negative control. Cells with brown-yellow granulations in endochylema or nuclei were considered as positive cells. According to the coloration, the cells were scored as following: 0, no staining; 1, faint-yellow; 2, brown-yellow; 3, dark-brown. Five fields of vision of each cover slip were observed under high power lens randomly. The cells were calculated and scored. The sum of the scores was divided by the number of cells, the result was considered as the expression intensity of survivin. The inhibition ratio of survivin expression was calculated with the following formula: inhibition ratio of survivin expression = (1-the expression intensity of survivin in the observation group/ the expression intensity of survivin in the blank control group)× 100%.

Radiation and apoptosis of PC-2 cells detected by flow cytometry

Cells were seeded in 25 cm^2 culture flasks and divided into blank control group, negative control group, positive experiment group, blank control + radiation group, negative control + radiation group, and positive experiment + radiation group. Each group contained 5 culture flasks. Transfection was performed the same as above. Twenty-four hours after the transfection, cells of groups 4, 5, 6 were radiated with 15 Gy of X-ray(6MV) at room temperature using a Varian 2300 C/D linear accelerator. The radiation conditions were ounce-skin distance = 100 cm, radiation dose rate = 2 Gy/min. After radiation, the cells were continuously cultured for another 24 h, digested, collected and washed twice with PBS. Then the cells were fixed with cold 75% ethanol at 4 ℃ overnight. The cells were centrifuged at 1000 r/ min for 5 min, the supernate was poured out, the cells were washed twice with PBS. The cells were resuspended with PBS containing 0.05 g/L RNase and incubated for 30 min at room temperature avoiding light to eliminate the intracellular RNA. The cells were centrifuged at 1000 r/min for 5 min, the supernate was poured out. The cells were resuspended with PBS containing 0.06 g/L propidium iodode again and incubated for 30 min at room temperature avoiding light, then cell apoptosis was detected by flow cytometry. The management was the same in cells of groups 1-3 except for no radiation.

Statistical analysis

Data were expressed as mean ± SD. All statistical analyses were performed using SPSS 10.0 software(one-way ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Verification of plasmids

The multiclone sites of plasmid Pgenesil-1 were as following: *Hin*dIII-*Xba*I-*Sal*I -*Pst*I-*Bam*HI-U6 Promotor-*Eco*RI-*Sal*I-*Xba*I-*Dra*III. An *Eco*RI site for plasmid Pgenesil-sur(+) and a *Sal*I site for plasmid Pgenesil-sur(-) were designed in the inserted fragments between the sites of *Bam*HI and *Hin*dIII. If the insertion was correct, a band about 400 bp should be cut off by *Eco*RI or *Sal*I. The results of digestion with restriction endonucleases and sequencing showed correct plasmids(Figure 1).

Results of transfection

Twelve hours after plasmid Pgenesil-sur(+) or plasmid Pgenesil-sur(-) was transfected into PC-2 cells using Lipofectamine™2000, expression of EGFP could be observed under an inverse fluorescence microscope. Twenty-four hours after the transfection, the expression of EGFP was the strongest. Seventy-two hours after the transfection, the expression of EGFP was attenuated gradually(Figure 2).

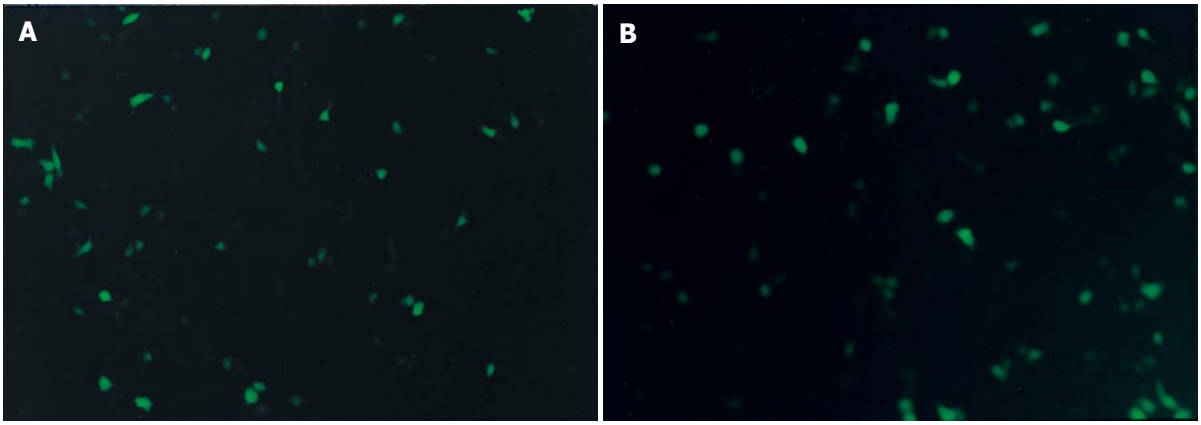

Figure 2 Expression of EGFP in PC-2 cells 24 h after trancfection of plasmid Pgenesil-sur(+) (**A**) and plasmid Pgenesil-sur(-) (**B**) (original magnification×200).

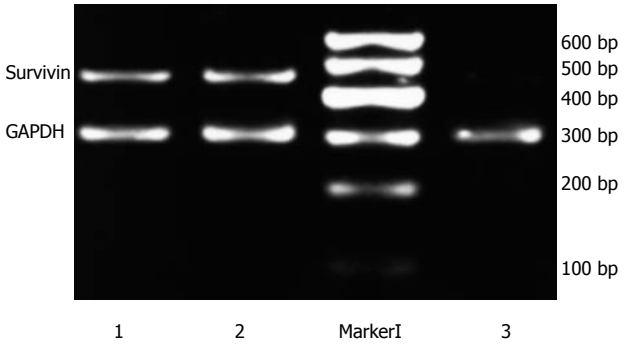

Figure 3 Down-regulation of survivin mRNA expression detected by semi-quantitive RT-PCR. 1: The blank control group; 2: The negative control group; M: Marker I; 3: The positive experiment group.

Significant down regulation of survivin mRNA expression by siRNA

Twenty-four hours after the transfection, the expression intensities of survivin mRNA in blank control, negative control and positive experiment groups were 0.96±0.02, 0.98±0.03 and 0.18±0.03, respectively. The statistical analysis showed that the expression of survivin mRNA in PC-2 cells was down regulated significantly after transfection with plasmid Pgenesil-sur(+) ($P<0.05$), the inhibition ratio was 81.25%. Then expression of PC-2 cells transfected with plasmid Pgenesil-sur(-) had no effect on the expression of survivin mRNA ($P>0.05$)(Figure 3).

Significant down regulation of Survivin protein expression by siRNA

Twenty-four hours after the transfection, the expression intensities of survivin protein in blank control, negative control and positive experiment groups were 2.29±0.23, 2.34±0.15 and 0.59±0.16, respectively. The statistical analysis showed that the expression of survivin protein in PC-2 cells was down- regulated significantly after transfected with plasmid Pgenesil-sur(+) ($P<0.05$), the inhibition ratio was 74.24%. The expression of PC-2 cells transfected with plasmid Pgenesil-sur(-) had no effect on the expression of survivin protein ($P>0.05$) (Figure 4).

Role of siRNA in inducing PC-2 cell apoptosis and enhancing its radiosensitivity detected by flow cytometry

Forty-eight hours after transfection, no cell apoptosis was detected in blank control and negative control groups, but in (7.03±1.12)% cells in the positive experiment group. The apoptosis rate in blank control+radiation group, negative control+radiation group and positive experiment +radiation group was (1.77±0.30)%, (1.66±0.46)% and (14.58±2.72)%, respectively. The statistical analysis showed that transfection with plasmid Pgenesil-sur(+) could induce spontaneous apoptosis of PC-2 cells in a certain degree and the effect was better than that of 15 Gy of X-ray radiation. Inhibiting the expression of survivin could enhance the radiosensitivity of PC-2 cells significantly. siRNA against survivin combined with radiation could induce significant apoptosis of PC-2 cells (Figure 5).

DISCUSSION

Pancreatic cancer is one of the most malignant tumors with a very poor prognosis. The 5-year survival rate of pancreatic cancer patients receiving surgery and chemotherapy ranges 1%-2%. One of the reasons for this low survival rate is the insensitivity of pancreatic cancer to most oncologic therapies such as chemotherapy, radiotherapy and immunotherapy. Tumor development and progression as well as resistance to most oncologic therapies result mainly from lacking response to apoptotic stimuli[13].

Survivin, a member of the IAP family, is a bifunctional protein that suppresses apoptosis and regulates cell division. Recent studies showed that there is a close relationship between survivin and malignant tumors. The expression of survivin is highly cancer-specific, and is one of the top four transcripts uniformly up-regulated in human cancers, but not in normal tissues[14]. The overexpression of survivin appears to correlate with aggressive tumor behavior and poor prognosis in colorectal cancer[15-17], neuroblastomas[18], melanomas[19], non small cell lung cancers[20], breast carcinomas[21], esophageal squamous cell carcinomas[22] and soft-tissue

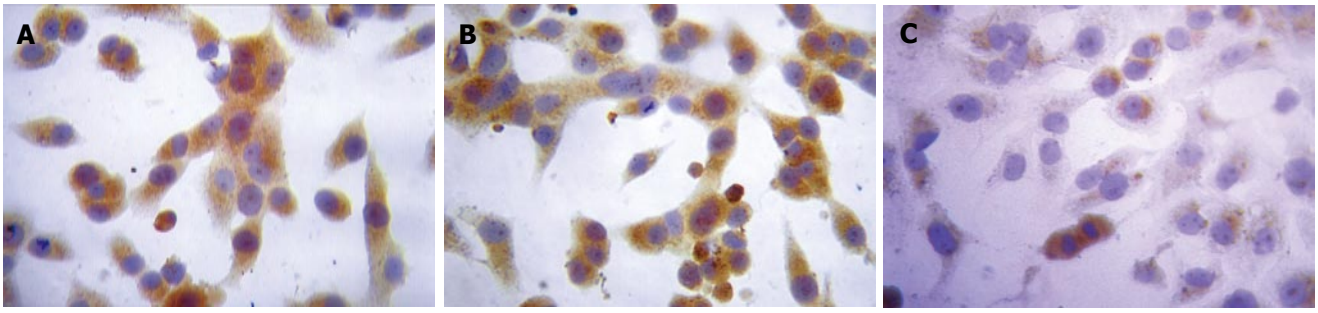

Figure 4 Down-regulation of survivin expression detected by immunohisto- chemical SP method in the blank control group (**A**), negative control group (**B**), and positive experiment group (**C**) (coloration with DAB, original magnification × 400)

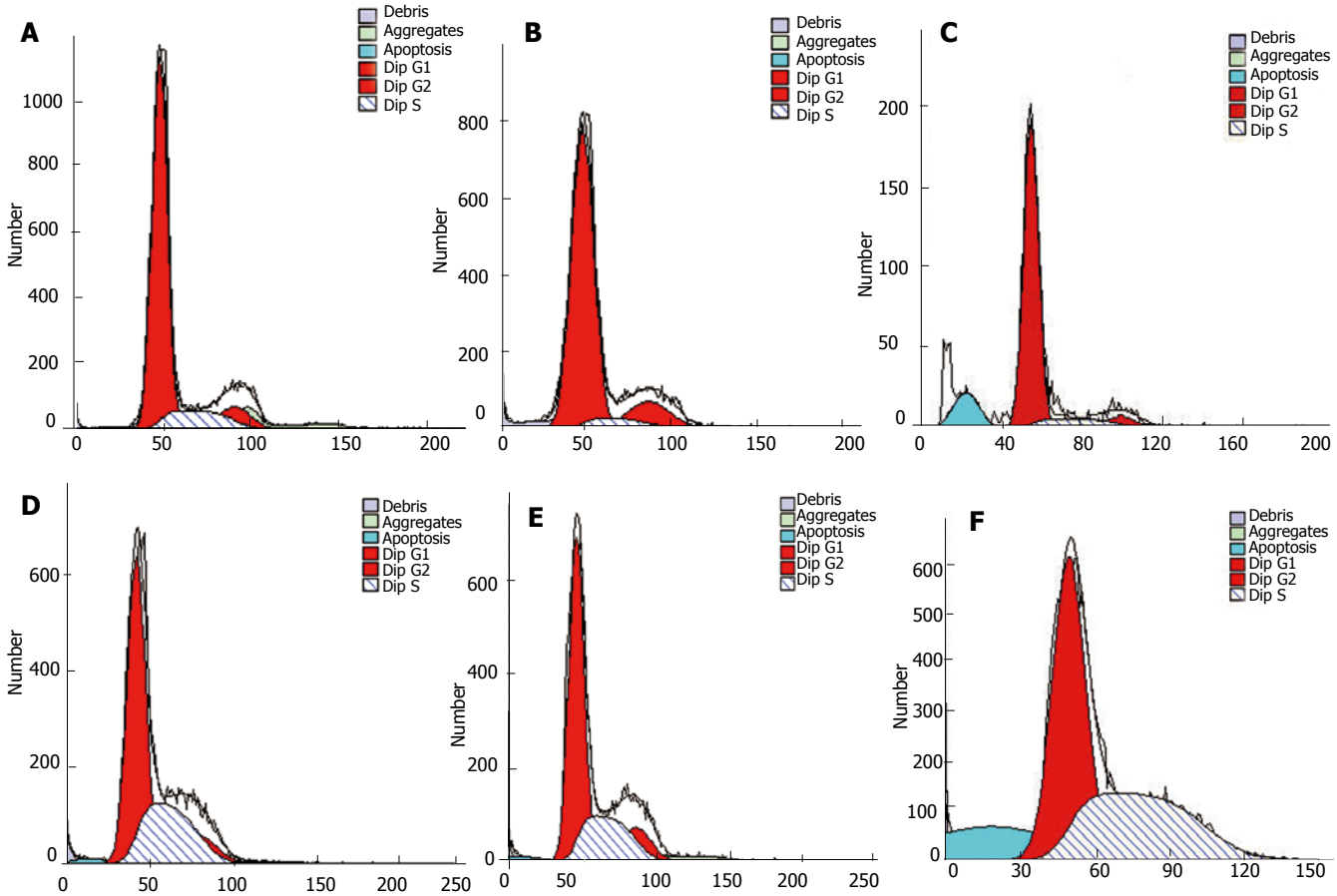

Figure 5 Apoptosis of PC-2 cells detected in 6 groups by flow cytometry (stained with PI)

sarcoma[23]. Additionally, survivin overexpression is also correlated with insensitivity to chemotherapy and radiotherapy in cancers[24,25]. Inhibiting the expression of survivin can induce tumor cell apoptosis, sensitize tumor cells to chemotherapy, radiotherapy and inhibit tumor-angiogenesis[26-29]. Survivin has become an ideal target for the diagnosis and treatment of cancer.

Studies on the relationship between the expression of survivin and pancreatic cancer showed that survivin is also overexpressed in pancreatic cancer, and participates in the development and progress of pancreatic cancer by inhibiting apoptosis. Its overexpression is associated with the insensitivity of pancreatic cancer to chemotherapy and radiotherapy, and is a poor prognosis marker[30-33]. All these studies suggest that survivin is of great potential value in the treatment of pancreatic cancer.

The mechanisms by which survivin inhibits cell apoptosis and cell division are still highly controversial. Survivin may regulate apoptosis by directly or indirectly inhibiting the activity of caspases (caspase-3, -7, -6, -8, -9, -10), or by interacting with cdk4 which releases P_{21} from its complex with cdk4 making it possible for P_{21} to complex with caspase-3, the initial step in inactivating caspase-3 in mitochondria. Survivin may possibly function to inhibit the activity of caspases indirectly *via* binding to and sequestering Smac/DIABLO, thus preventing Smac/DIABLO binding to other IAPs. Survivin might be an additional chromosomal passenger protein just like inner

centromere protein (INCENP), TD-60 and Aurora-B, and is important for cytokinesis and chromosome movement during cell division. Overexpression of survivin may overcome the G2/M phase checkpoint to enforce progression of cells through mitosis[1].

RNAi is characterized by high efficiency, high specificity and low toxicity. siRNA has become a powerful tool for studies on gene function, cancer and viral disease therapy. In this study, we designed and constructed the siRNA plasmid expression vector. Its transcript can form a short hairpin RNA(shRNA) with inverted repeat sequence separated by a short loop sequence, then the shRNA is processed into functional siRNA to degradate target mRNA and silences its expression. The siRNA plasmid expression vector constructed in this study contains the EGFP gene that makes it convenient to observe the result of transfection. By aiming at different sequence of the target gene, the effect of RNAi may be greatly different [34, 35] suggesting that it is very important to find the proper sequence. The sequence of Kappler consulted in this study has been confirmed effective in multiple tumor cell lines[12,36]. The results of our studies also demonstrated that it was effective both in pancreatic cancer cell line PC-2 and in breast cancer cell line MCF-7 (data not shown), suggesting that this sequence may be of universality in malignant tumors and should be a preferential sequence when study on the RNAi against surviving is carried out.

In this study, we investigated whether the sensitivity to X-irradiation could be affected by inhibiting survivin expression with siRNA. The results showed that the siRNA against survivin plasmid expression vector constructed in this study had highly specificity, and could suppress the expression of survivin in PC-2 cells efficiently suggesting that inhibiting the expression of survivin can induce spontaneous apoptosis of pancreatic cancer cells in a certain degree and enhance its radiosensitivity significantly. siRNA against survivin is of potential value in the treatment of pancreatic cancer.

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BASIC RESEARCH

Hyperlipidemia intensifies cerulein-induced acute pancreatitis associated with activation of protein kinase C in rats

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Abstract

AIM: To investigate the effects of hyperlipidemia on acute pancreatitis (AP) and the possible mechanisms.

METHODS: Rat models of hyperlipidemia and AP were established by Triton WR1339 and cerulein respectively. Human albumin was used to treat AP complicated by hyperlipidemia. In each group, we compared the histological score, volume of ascites, ratio of pancreatic wet/dry weight, serum amylase (AMY) and pancreatic acinar cell apoptosis. The level of protein kinase C (PKC) membrane translocation in pancreatic tissue was detected by Western blot.

RESULTS: In the hyperlipidemia model established by Triton WR1339, triglyceride (TG) increased remarkably and reached its peak 6 h after injection, and most rats developed mild acute pancreatitis. Histological score, volume of ascites, ratio of wet/dry weight and serum AMY in AP animals with hyperlipidemia were obviously higher than those in AP animals ($P < 0.05$) and decreased after albumin therapy but not significantly ($P > 0.05$). Apoptotic cells detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) increased in AP animals with hyperlipidemia and did not change distinctly after albumin therapy. PKC membrane translocation level increased in AP animals with hyperlipidemia and decreased remarkably after albumin therapy ($P < 0.05$).

CONCLUSION: Hyperlipidemia may induce AP or intensify pancreatic injury. Albumin therapy can not alleviate pancreatic lesion effectively. PKC activation may be one mechanism by which AP is intensified by hyperlipidemia.

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INTRODUCTION

Acute pancreatitis (AP) is a common disease that normally runs a benign course in the majority of patients. However in up to 20% of individuals the disease is severe and may be associated with a mortality of about 20%[1,2]. Since Speck in 1865[3] noted the association between hyperlipidemia and AP, their relationship has been studied by many scholars. Currently, hyperlipidemia is another main cause of AP besides cholelithiasis and alcohol abuse[4]. Serum triglyceride (TG) levels higher than 1000 mg/dl seem to be a serious factor for developing AP, particularly in patients suffering from familial hyperlipidemia (types 1, 4 or 5 according to Fredrickson's classification). AP occurs with an incidence of up to 21%[5,6]. Hydrolysis of TG by pancreatic lipase with the local release of large quantities of free fatty acids (FFAs) has been proposed as the pathogenic mechanisms. Hyperlipidemia is a common cause of AP. Moreover, lipid levels increase above normal in up to 50% of patients with AP of any cause[7].

Several studies in humans and animals have been performed to evaluate hyperlipidemia as a risk factor for developing AP. However, there is no ideal animal model of hyperlipidemic AP. Information about the influence of hyperlipidemia on the course of AP is relatively rare. The majority of investigators considered that hyperlipidemia can intensify the pancreatic lesion and systemic inflammatory response. Saharia *et al*[8] perfused the pancreas preparations with TG and FFAs (oleic acid) and found that they can lead to edema of the isolated pancreas, weight gain, and elevation of serum AMY. The injury caused by FFAs is similar to that seen with TG infusion, but it occurs more rapidly. These studies add support to the concept that hypertriglyceridemia can initiate pancreatic injury and the release of FFAs may be the mechanism. Kimuru *et al*[9] initiated an AP model with cerulein, retrograde duct injection of sodium taurocholate,

bile/pancreatic duct respectively and found that the isolate pancreatic damage is increased by TG perfusion. However, some reports suggest that hyperlipidemia could not aggravate the course of AP. Paye *et al*[10] prepared an AP model with cerulein and injected TG simultaneously, and found that histological alterations, pancreatic enzyme level and ascites in these models do not differ from those in AP patients without TG injection. To approve this result further, the authors collected very low density lipoprotein (VLDL) from AP patients and found that the VLDL intravenous infusion for hypertriglyceridemia does not alter the course of AP[11]. Therefore the role of hyperlipidemia in acute pancreatitis seems questionable.

The mechanism by which hyperlipidemia leads to AP is still unclear. A well-accepted mechanism proposed by Havel *et al*[12] is that unbound FFAs are toxic and could produce acinar cells or capillary injury. Increased concentration of chylomicrons in pancreatic capillaries causes capillary plugging and leads to ischemia and acidosis. FFAs can induce activation of trypsinogen and initiate AP. Recently, it was reported that FFAs are toxic to pancreatic β-cells leading to diabetes and insulin resistance syndrome. The toxicity is associated with abnormalities in the insulin signaling cascade and may be mediated by FFA activation of protein kinase C (PKC)[13]. Also, there is abundant PKC in pancreatic acinar cells[14]. Other similar mechanisms of FFAs may damage acinar cells.

The aim of the present work was to determine whether endogenous hyperlipidemia aggravates *in vivo* cerulein-induced edematous AP and activates PKC in pancreas of rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 250-270 g were used. Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rats were maintained at 23 ℃ in a 12 h light dark cycle, with free access to water and standard rat chow. The rats were fasted for 12 h prior to the experiment, but with free access to water. They were anesthetized with chloral hydrate (300 mg/kg intraperitoneal injection). Body temperature was monitored and maintained at 37.5 ± 1 ℃ using a heating device.

Models of hyperlipidemia, AP, and AP with hyperlipidemia

Hyperlipidemia was induced by intravenous injection of 10% Triton WR1339 solution (TyloxAPol, Sigma, USA) through the left external iliac vein. Triton WR1339, a non-ionic surface active detergent leads to an increase of endogenous TG and total cholesterol (TCH) blood concentrations[15, 16]. We determined the required amounts of Triton WR1339 with two doses of 100 mg/kg and 400 mg/kg body weight respectively. A volume of 400 mg/kg Triton WR1339 solution could induce maximal hyperlipidemia. The non-hyperlipidemic animals received an injection of physiological saline solution intravenously instead of Triton WR1339.

AP was induced by 4 h abdominal subcutaneous injection of cerulein (Sigma, American, 20 μg/kg in 0.5 mL of saline vehicle) as previously described[17].

To explore the influence of hyperlipidemia on acute experimental pancreatitis, we used five different groups of animals with 12 rats in each group. The animals of the first group [$C_{(sal)}$] received 1.0 mL saline (sal) solution, and the second group [$C_{(tri)}$] received 1.0 mL of 10% Triton WR1339 (tri) intravenously. In these two groups [$C_{(sal)}$ and $C_{(tri)}$] no pancreatitis was induced. AP was induced in the third group (AP) and the fourth group (HAP) as described above. The animals of group HAP received an injection of 1.0 mL of Triton WR1339 intravenously 6 h before pancreatitis was induced with cerulein infusion. Since albumin could effectively combine FFAs in circulation to prevent cellular damage[18], in the fifth treatment group (THAP), the rats received an injection of 1.25 g/kg albumin intravenously after pancreatitis was induced.

Analysis of serum lipid, AMY activity and histology

Blood samples were taken from all animals before and 3, 6, 9 h after induction of hyperlipidemia or AP with hyperlipidemia. The samples were used to measure TG, TCH, AMY activity.

Three and six hours after induction of AP, the rats were sacrificed by aortic puncture and blood samples taken for plasma biochemical measurements. A complete macroscopic examination of the peritoneal cavity and ascites was immediately performed, and the pancreas was removed using a standardized monobloc surgical technique. The pancreas was divided into three parts. The pancreatic head was fixed in Bouin's solution, embedded in paraffin, sliced, and stained with hematoxylin and eosin. The pancreas body was put into liquid nitrogen for PKC analysis. To better estimate the amount of edema, the remaining pancreatic tissue was weighed and dessicated for 24 h at 173 ℃ to measure its dry weight. We used the wet to dry ratio to quantify edema formation.

To quantify the histological changes, two pathologists judged the tissue samples using a point score as previously described[19,20]. Plasma AMY concentrations were determined after plasma dilution (1/20) by the colorimetric assay AMYL (Boehringer Mannhein, Germany). Plasma TG was measured using the colorimetric assay TG GPOPAP (Boehringer).

Detection of apoptosis

Recent studies indicate that mild acute pancreatitis is related to the extent of acinar cell apoptosis[21-23]. A pancreatic tissue section was prepared and assayed with the *in situ* cell death detection kit, POD (Boehringer Mannheim, Germany) following the manufacturer's instructions. Slides were counterstained with hematoxylin and viewed under a light microscope.

Activation of protein kinase C

PKC activation was determined in both cytosolic and particulate fractions as previously described[24]. Frozen pancreatic tissue(50 mg) was rapidly thawed and homogenized at 4 ℃ in lysis buffer A (mM: Tris-HCl 50; pH 7.4; NaCl 150; EDTA 2; EGTA 2; DTT 2; Na py-

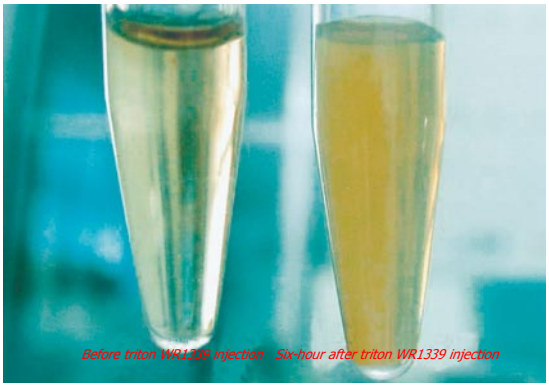

Figure 1 Development of chylomicronemia after Triton WR1339 injection.

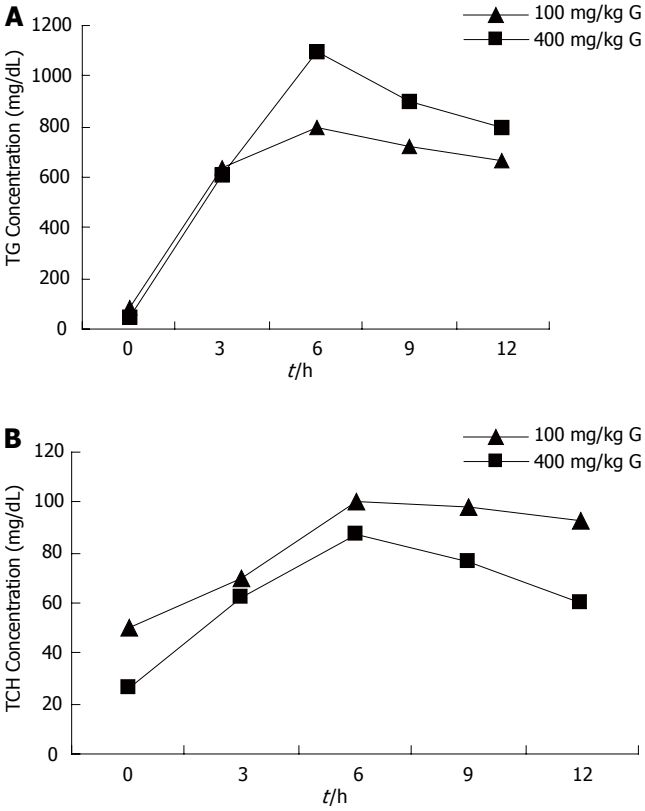

Figure 2 Curves of serum TG and TCH induced by Triton WR 1339 in rats.

rophosphate 5; KF 50; PMSF 1; 1μg/mL: Aprotinin, Leupeptin, Pepstatin A). Homogenates were centrifuged at 100 000 r/min for 30 min at 4 ℃ in an Optima TLX ultracentrifuge to yield cytosol. The pellet fractions were resuspended in 100 μL of buffer A containing 0.5% Nonidet *P*-40, sonicated and centrifuged again. The resulting supernatants taken as the particulate fractions with the cytosolic fractions were assayed for PKC immunoreactivities. Throughout the study, protein concentrations were determined by the Lowry's method with bovine serum albumin as standard. Aliquots of the cell lysates containing 100 g of total protein were resolved in 10% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences, Inc.). The membranes were blocked in TBS-Triton-X 100 (0.2% v/v, pH 7.4) containing 5% milk for 2 h at room

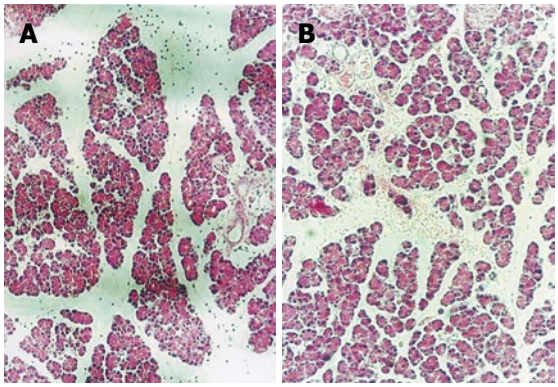

Figure 3 Microscopic pancreatic changes in rats with AP induced by cerulean (**A**) and hyperlipidemic AP induced by cerulein and Triton WR1339 (**B**) 6 h after injection (HE × 200).

temperature followed by incubation with PKC antibody at the dilution described by manufacturer's instructions at 4 ℃ overnight. The membranes were incubated with anti-goat horseradish peroxidase complex (diluted 1:2000) for 2 h at room temperature. The proteins on the membranes were exposed to X-ray films at multiple time points and visualized by ECL detection system (Amersham Biosciences, Inc.).

Statistical analysis

Data were expressed as mean ± SD and analyzed using Student's *t* test or Student's Newman Keuls-test by SPSS 11.0 software. Quantitative analysis for immunoblot was done by scanning the x-ray film and determined using the Fotodyne Gel-Pro analyzer program.

RESULTS

Changes of serum lipid, AMY and pathology of pancreas in $C_{(tri)}$ group

The animals receiving 100 mg/kg and 400 mg/kg Triton WR1339 had high TG and TCH. The serum TG increased most obviously and reached its peak 6 h after Triton WR1339 infusion with induction of chylomicronemia (Figure 1). The TG value increased 10-fold and 20-fold after 100 mg/kg and 400 mg/kg Triton WR 1339 infusion respectively, whereas the serum TCH increased only 2-fold and 3-fold (Figure 2). The serum AMY activities did not significantly increase after induction of hyperlipidemia ($P > 0.05$). The animals did not demonstrate ascites and pancreatic edema in macroscopy. Nine rats (9/12) showed mild pancreatic edema, whereas no histological changes occurred in the saline-treated animals (group $C_{(sal)}$).

Effects of hyperlipidemia on pancreatic histology in AP rats

The pancreatic changes included interstitial edema, increased interlobular interstice and infiltration of some inflammatory cells in the animals 3 h after cerulein infusion. The pancreas of AP animals 6 h after cerulein injection had serious edema with tensioned capsules and separated small lobuli. Wide lobular and acinar interstices, anamorphic acinar structure, intracytoplasmic vacuolization and

Table 1 Effects of caerulein and albumin therapy on histological pancreatic lesions, volume of ascites and ratio of pancreatic wet/dry weight (mean ± SD)

Group	Time	Histological score	Ascites(ml)	Wet/dry
C(sal)	3 h	0.33 ± 0.52	0	1.35 ± 0.09
	6 h	0.33 ± 0.52	0	1.35 ± 0.09
AP	3 h	3.50 ± 1.38[a]	0.87 ± 0.58	1.67 ± 0.05[a]
	6 h	5.33 ± 1.03[a]	1.58 ± 0.92	1.76 ± 0.14[a]
HAP	3 h	5.17 ± 1.17[c]	2.08 ± 0.58[c]	1.95 ± 0.44
	6 h	6.83 ± 1.16[c]	4.12 ± 1.58[c]	2.15 ±0.39
THAP	3 h	4.83 ± 1.16	1.87 ± 0.93	1.72 ± 0.14
	6 h	6.67 ± 1.21	3.68 ± 1.42	1.88 ± 0.33

[a]$P < 0.05$ *vs* control group, [c]P <0 .05 *vs* caerulein group.

Table 2 Effects of albumin therapy on serum TG, TCH and AMY in rats (mean ± SD)

Group	Time	TG	TCH	AMY
C(sal)	-	47.00 ± 23.38	26.25 ± 2.36	1658.50 ± 335.21
AP	3 h	63.66 ± 11.52	61.00 ± 6.00	5128.66 ± 259.65
	6 h	72.66 ± 22.65	67.00 ± 8.71	6641.00 ± 213.75
C(tri)	3 h	899.51 ± 240.52	76.03 ± 16.14	1652.55 ± 300.46
	6 h	797.07 ± 356.18	60.51 ± 33.49	1253.72 ± 254.80
HAP	3 h	1604.16 ± 248.10[a]	264.33 ± 50.33[a]	18390.00 ± 7158.77[a]
	6 h	1506.66 ± 327.72[c]	230.33 ± 31.87[c]	11611.66 ± 4513.67[c]
THAP	3 h	1396.83 ± 658.65	221.00 ± 60.46	20473.33 ± 12005.46
	6 h	1167.33 ± 228.28	211.00 ± 14.02	16524.39 ± 5273.52

[a]$P < 0.05$, [c]$P < 0.05$ *vs* triton group.

infiltrated inflammatory cells were observed (Figure 3). After 3 h, the pancreatic damage was intensified in the animals of group HAP, and some apoptotic bodies (inclusion) and more inflammatory cells appeared. There were scattered erythrocytes and inflammatory cells, distended small veins in the pancreas 6 h after HAP model was induced (Figure 3). There were no pathological changes after albumin therapy.

Pancreatic histological score, volume of ascites and pancreatic wet/dry ratio in rats

The animals receiving cerulein (group $C_{(tri)}$) had significantly ($P < 0.05$) higher pancreatic histological score, volume of ascites and pancreatic wet/dry ratio than the control group. The pancreatic histological score, volume of ascites and pancreatic wet/dry ratio were significantly higher ($P < 0.05$) in the AP animals with hyperlipidemia (group HAP) than in the animals receiving only cerulein infusion (group $C_{(tri)}$). After albumin treatment (group THAP) these parameters decreased slightly but were higher in the 6 h group than in the 3 h group ($P > 0.05$). These results suggested that hyperlipidemia could aggravate pancreatic edema of the AP animals and albumin therapy was not obviously effective (Table 1).

Serum TG, TCH and AMY in rats

The serum TG and TCH concentration increased 1-2 folds in the cerulein-induced AP animals. There were no statistical differences between group AP and group $C_{(sal)}$ ($P > 0.05$). The hyperlipidemic AP animals had significantly higher serum TG and TCH values than the rats receiving Triton WR1339 (group $C_{(tri)}$) injection. The serum TG and TCH concentration in the animals with albumin therapy (group THAP) decreased slightly. The AMY activities in the hyperlipidemic group $C_{(tri)}$ remained unchanged throughout the investigation, and increased obviously ($P < 0.05$) in the AP group. In comparison with the AP group, the AMY values of group HAP and THAP were significantly higher ($P < 0.05$). This result demonstrated that AP could induce mild hyperlipidemia. Albumin could not decrease the circulating lipid and AMY concentration (Table 2).

Apoptosis of pancreatic cell in rats

It was reported that the extent of acinar cell apoptosis is related to the severity of mild AP[22]. Careful biochemical and morphological examination of experimental models of AP has shown that severe AP is associated with necrosis, whereas mild acute pancreatitis is associated with apoptotic cell death[20,21]. In our study, the TUNEL methods was used for *in situ* apoptotic acinar cell identification. The apoptotic cells were not visible in the pancreas of control group, but a few apoptotic cells (brown) were visible in cerulein-induced AP animals. A subpopulation of apoptotic cells scattered throughout the tissue section was stained brown Figure 4, but the apoptotic cells did not markedly decrease after albumin therapy.

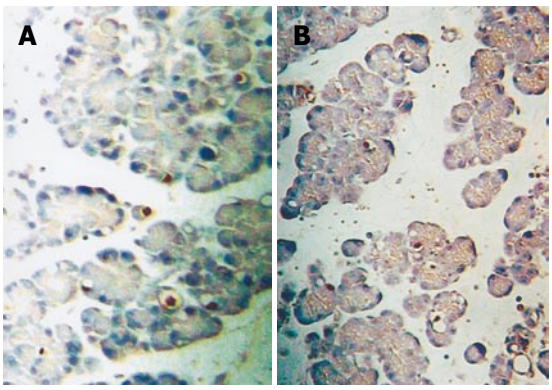

Figure 4 Detection of apoptotic cells by TUNEL in rats with AP induced by cerulein (**A**) and hyperlipidemic AP induced by cerulean and Triton WR1339 (**B**) counterstained with hematoxylin (H × 200).

Translocation of PKC in pancreatic tissue

To confirm the activation of PKC, we examined the translocation of the enzyme from the cytosol to the particulate membrane by Western blot. The cytosolic PKC gradually decreased in the control group $C_{(sal)}$, hyperlipidemic group $C_{(tri)}$, cerulein-induced AP group and HAP group. After albumin treatment the cytosolic PKC increased remarkably in group THAP. However, the particulate PKC gradually increased in the above four groups and decreased after albumin therapy (Figure 5). The degree of PKC translocation was evaluated by the proportion of particulate PKC in the total PKC as previously described[25]. The translocation rate of PKC was significantly higher ($P < 0.05$) in hyper-

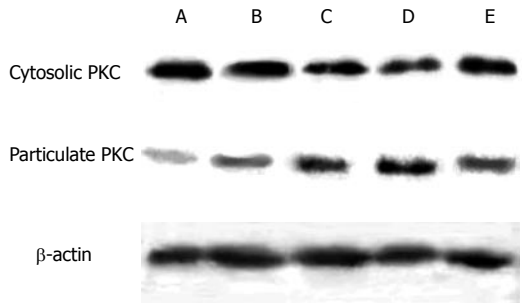

Figure 5 Effects of hyperlipidemia, AP, AP accompanying hyperlipidemia and albumin therapy on PKC membrane translocation detected by Western blot in rats of control group (A), Triton group (B), caerulein group (C), Triton+caerulein group (D), Triton+caerulein+albumin group (E).

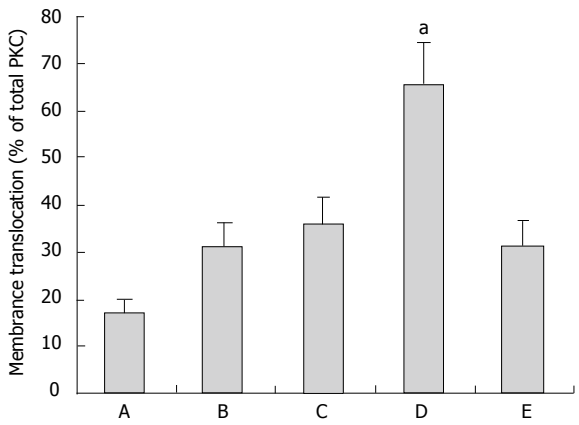

Figure 6 Effects of Triton hyperlipidemia, caerulein pancreatitis, AP with hyperlipidemia and albumin therapy on PKC membrane translocation in rats. Quantitative analysis of PKC membrane translocation level was detected by Western blot. $^aP < 0.05$ Triton+caerulein group *vs* caerulein and Triton+caerulein+albumin group ($n = 6$).

lipidemic AP animals (group HAP) than in non-hyperlipidemic AP rats (group AP)(Figure 6). In the hyperlipidemic AP group, the translocation rate of PKC was significantly decreased ($P < 0.01$) after albumin treatment. This result implied that hyperlipidemia could induce pancreatic PKC activation in normal and AP rats, which was consistent with the pancreatic injury. Albumin could reduce hyperlipidemia-induced PKC activation.

DISCUSSION

Hypertriglyceridemia is an etiological factor in patients with AP while TG level exceeding 1000 mg/dL or lactescent serum is a necessary condition for hyperlipidemic AP diagnosis[26]. Triton WR1339 could increase serum TG and is an ideal drug to establish hyperlipidemic animal model with TG elevation.

The rats without pancreatitis showed no clinical differences after Triton WR1339 infusion compared with saline infusion. Macroscopically, the pancreas of the hyperlipidemic and normal animals showed no difference. Microscopically, the animals treated with Triton WR1339 showed smooth edema, whereas no changes occurred in the non-hyperlipidemic animals, suggesting that severe hyperlipidemia may cause mild AP. The serum AMY activities were also slightly increased after hyperlipidemia induction, but there was no significant difference ($P > 0.05$). To investigate the effect of hyperlipidemia on AP, we established the AP model when the endogenous hyperlipidemia induced by Triton WR1339 reached its maximal value. Theoretically, significant influence of hyperlipidemia on AP would appear. We produced the hyperlipidemic AP animal model by injecting cerulein 6 h after Triton WR1339 infusion. It was reported that albumin could effectively alleviate the toxicity of TG to pancreatic cells[18]. A medium dose of albumin was injected through external iliac vein in the hyperlipidemia AP animals to observe the changes of pancreatic damage. As a result, hyperlipidemia aggravated pancreatic edema, volume of ascites and pancreatic cell apoptosis. The serum lipid in the hyperlipidemic AP animals increased compared with that in the hyperlipidemic animals, suggesting that hyperlipidemia may aggravate the course of AP while AP may exacerbate hyperlipidemia. There is a vicious circle between hyperlipidemia and AP. Accordingly, it is very important to decrease circulating lipid in clinical patients with AP. In our studies, the pancreatic damage aggravated by hyperlipidemia was not prevented by simultaneous perfusion of albumin, which might be due to the fact that the concentration of albumin in the inflamed pancreas may be too low or there may be other mechanisms of pancreatic injury. It was reported that FFAs could inhibit division and proliferation of pancreatic endocrine cells by activating PKC and induce apoptosis of pancreatic β-cells. As a result insulin secretion is decreased and diabetes occurs[27]. There is abundant PKC in pancreatic acinar cells[14]. Our study showed that FFAs could cause injury of pancreatic exocrine cells. PKC activity of pancreas increased in the hyperlipidemia and AP animals. The PKC activity changes were consistent with the degree of pancreatic injury, suggesting that PKC may participate in the course of hyperlipidemia inducing or aggravating pancreatic injury of AP. The PKC activity in hyperlipidemic AP animals was decreased significantly ($P < 0.01$) after albumin therapy, which implies that albumin may reduce the PKC activation through binding to FFAs. Although PKC activation decreased obviously ($P < 0.01$) after albumin therapy, the pancreatic injury was slightly relieved ($P > 0.05$), suggesting that PKC activation may be an important aspect of hyperlipidemia aggravating AP, and there may be other mechanisms of hyperlipidemia-induced AP.

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RAPID COMMUNICATION

Oxygen radical formation does not have an impact in the treatment of severe acute experimental pancreatitis using free cellular hemoglobin

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Abstract

AIM: Microcirculatory dysfunction and free oxygen radicals are important factors in the pathogenesis of severe acute pancreatitis. Additional oxygen delivery might enhance lipid peroxidation but may also improve pancreatic microcirculation. This study assesses the effect of free cellular bovine hemoglobin on the formation of oxygen radicals and microcirculation in a rodent model of severe acute pancreatitis.

METHODS: Fifteen minutes after induction of acute pancreatitis Wistar rats received either 0.8 mL bovine hemoglobin (HBOC-200), hydroxyethyl starch (HES) or 2.4 mL of normal saline to ensure normovolemic substitution. After 6 h of examination the pancreas was excised and rapidly processed for indirect measurement of lipid peroxidation products malondialdehyde (MDA) and reduced glutathione (GSH) in pancreatic tissue.

RESULTS: The single application of HBOC-200 improved pancreatic microcirculation and reduced histopathological tissue damage significantly. Tissue concentration of MDA did not differ between the groups. Also no differences in GSH levels were detected.

CONCLUSION: Though the single application of HBOC-200 and HES improve pancreatic microcirculation, no differences in lipid peroxidation products were detected. The beneficial effect of additional oxygen supply (HBOC-200) does not lead to enhanced lipid peroxidation.

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Key words: Hemoglobin-based-oxygen-carrier; HBOC; Blood substitutes; Severe acute pancreatitis; Free oxygen radicals; Oxidative stress

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INTRODUCTION

Microcirculatory dysfunction and free oxygen radicals are important factors in the pathogenesis of acute experimental pancreatitis[1-3]. Oxygen free radicals are formed during aerobic cellular metabolism. Under physiological conditions they are eliminated by a system of enzymatic and non-enzymatic antioxidants. Under conditions of imbalance like in acute pancreatitis between antioxidants and oxidants as in hyperoxygenation, ischemia and reperfusion and tissue inflammation "oxidative stress" occurs[4-8].

Oxygen species initiate a pronounced peroxidation of membrane lipids, detectable after 30 min by a significant rise in the lipid peroxidation marker malondialdehyde (MDA), persisting up to 16 h[8,9]. At the same time a significant decrease in glutathione thiols (GSH, potent antioxidant in the pancreas) occurs[10,11]. In the human, acute pancreatitis increases the amount of lipid peroxidation products and decreases antioxidants[12-14].

It has been shown that pancreatic microcirculatory deficits can be treated with bovine haemoglobin, which improves fluidity due to its colloid effects and delivers cell free oxygen to oxygen deprived tissue[15]. However, additional application of cell free oxygen might potentially enhance detrimental O_2 radical products[16], which could limit the positive value of bovine haemoglobin in the treatment of severe acute pancreatitis.

This study assesses the relationship between free oxygen radicals and pancreatic microcirculation in a rodent model of severe acute pancreatitis after therapeutic application of a hemoglobin based oxygen carrier (HBOC).

MATERIALS AND METHODS

The experimental protocol was approved by the Ethical

Committee of the Hamburg Federal Board of Veterinary Medicine and Animal Care. After overnight fasting with free access to water containing 20% glucose, thirty female Wistar rats (230-250 g) were randomly assigned to three groups (n = 10). Anesthesia was induced by intraperitoneal pentobarbital (40 mg/kg BW) and ketamine (10 mg/kg BW) allowing spontaneous breathing by tracheostomy. Ringer lactate was infused for fluid resuscitation keeping the central venous pressure between 4 and 6 mmHg and heart rate as well as mean arterial pressure within 10% of baseline frequency throughout the experiment (Datex AS/3 monitoring system; Hoyer, Bremen, Germany).

Acute pancreatitis was induced according to a standardised regimen[17] using a combination of intravenous cerulein infusion (5 mg/kg per hour; Takus, Pharmacia, Erlangen, Germany) at a rate of 1 mL/h over 6 h and intraductal infusion of glycodeoxycholic acid (GDOC: 10 mmol/L; 1 mL/kg for 300 s; infusion pressure 25 to 30 mmHg).

Fifteen minutes after induction the animals received either 0.8 mL bovine hemoglobin (HBOC-group) (HBOC-200, Biopure MD, USA), 0.8 mL hydroxyethyl starch (HES-group 3) or 2.4 mL of normal saline (NaCl-group) to ensure normovolemic substitution. *In vivo* fluorescence microscopy was performed with an epi-illumination unit (Zeiss, Germany). Microcirculation was observed for 6 h, measurements were performed in the head of the pancreas: 0 min was defined as the intraductal infusion of GDOC. Leucocyte adherence (area of adherent leucocytes in a percentage of the vein cross section) and functional capillary density (number of perfused capillaries to the total number of capillaries) were studied. After 6 h of examination the animals were sacrificed by an intravenous injection of pentobarbital. Pancreatic tissue samples were excised for determination of tissue damage and malondialdehyde (Lipid Peroxidation Assay Kit, Calbiochem, San Diego, USA) and GSH concentrations (Glutathion Assay Kit, Calbiochem, San Diego, USA) specified in μmol/L/g protein. The samples were immediately processed and stored at -80 ℃. The Glutathione Assay Kit takes advantage of a 2-step reaction. The first step involves the formation of thioethers when a patented reagent reacts with all mercaptans (including glutathione) in the sample. The second step is an elimination reaction that takes place in alkaline conditions. This second reaction is mediated by 30% NaOH which specifically transforms the substitution product obtained with GSH into a chromophoric thione with a maximal absorbance at 400 nm. The main advantage of the method is that it does not require any enzyme as a reagent.

The Lipid Peroxidation Assay Kit takes advantage of a chromogenic reagent (10.3 mmol/L N-methyl-2-phenylindole, in acetonitrile) which reacts with MDA and 4-hydroxyalkenals at 45 ℃. Condensation of one molecule of either MDA or 4-hydroxyalkenal with 2 molecules of 10.3 mmol/L N-methyl-2-phenylindole yields a stable chromophore with maximal absorbance at 586 nm.

Tissue protein concentration was detected by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colometric detection and quantitation of total protein. This method combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the colometric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchonic acid[18].

The histopathological scoring was analyzed by light microscopy using a validated score quantifying acinar necrosis, fat necrosis and hemorrhage, leucocyte infiltration and edema[19].

Statistical analysis

Statistical analysis of the changes from baseline values in each group of animals utilized the paired Student's t-test. Significances between the groups were assessed by the unpaired t-test. $P < 0.05$ was considered significant. All data are presented as mean ± SD.

RESULTS

Results of pancreatic microcirculation and tissue damage have been reported elsewhere[15].

Microcirculation[15]

In essence pancreatic microcirculation improved in the HBOC-200-group when compared to NaCl-group [functional capillary density (fcd): 0.7 (SD 0.3) *vs* 0.33 (0.12); $P < 0.05$]; leucocyte adherence (la): 0.27 (0.14) *vs* 0.54 (0.19); $P < 0.05$). HES also improved pancreatic microcirculation when compared to NaCl-group (fcd: 0.49 (0.15) *vs* 0.33 (0.12); $P < 0.05$ and la: 0.39 (0.11) *vs* 0.54 (0.19); $P < 0.05$).

Tissue damage[15] (Figures 1-4)

The histological score showed significantly less tissue damage in HBOC-group (Figure 1) versus NaCl-group (6.25 *vs* 9.25) (range 3-8.5 *vs* 8-10.75); $P < 0.001$. The overall score is reflected within all subgroups. Comparing HES and HBOC-therapy significantly less tissue damage was found (6.25 *vs* 8) (range 3-8.5 *vs* 6.5-10.25); $P = 0.006$ in the HBOC group. When comparing HES (Figure 2) and NaCl (Figure 3) less necrosis and hemorrhaging (1.75 *vs* 2.75) (range 1.5-2.75 *vs* 2.25-3.5) was found in the HES therapy group(Figure 4).

Oxygen radicals

Tissue concentration of MDA did not differ between the groups (HBOC-200-group: 4.37 ± 1.11 μmol/L per g protein; HES-group: 3.77 ± 1.45 μmol/L per g protein; NaCl-group: 4.31 ± 1.19 μmol/L per g protein. MDA-tissue levels (n = 10) of sham operated rats were 5.41 ± 2.12 μmol/L per g protein.

Also no differences in GSH levels were detected HBOC-group: 2.93 ± 1.72 μmol/L per g; HES-group: 4.46 ± 3.55 μmol/L per g; NaCl-group: 3.7 ± 3.5 μmol/L per g. Control GSH-tissue levels (n = 10) of sham operated rats were 12.19 ± 7.04 μmol/L per g protein (Figure 5).

DISCUSSION

Clinical investigations with free hemoglobin solutions have

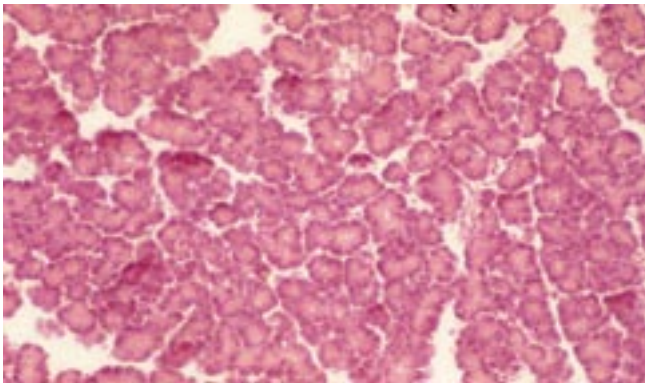

Figure 1 Light microscopy (display alternation = 200) of pacreatic tissue 6 h after induction of acute pancreatitis and traement with HBOC (score: 6 points out of 16).

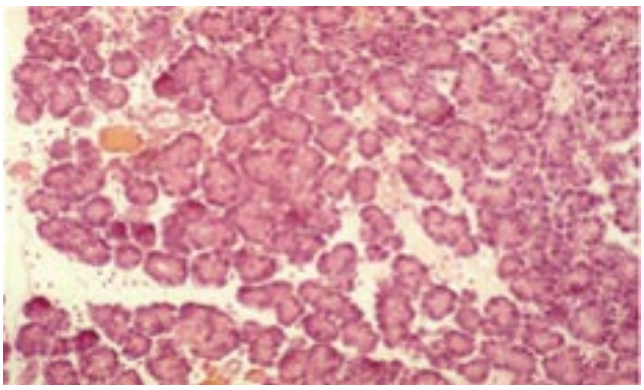

Figure 2 Light microscopy (display alternation = 200) of pacreatic tissue 6 h after induction of acute pancreatitis and traement with HES (score: 8.5 points out of 16).

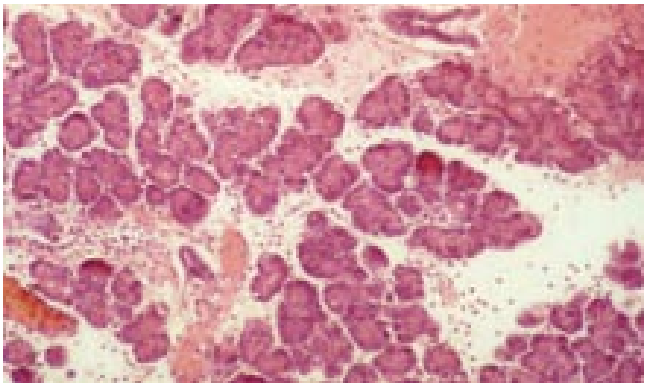

Figure 3 Light microscopy (display alternation = 200) of pacreatic tissue 6 h after induction of acute pancreatitis and treatment with NaCl (score: 10 points out of 16).

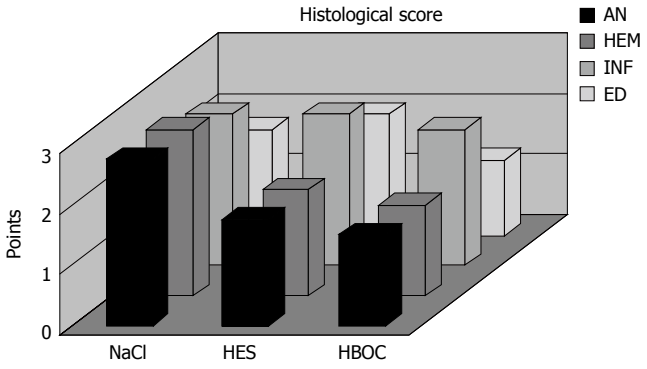

Figure 4 Histological scoring of pancreatic tissue 6 h after induction of acute pancreatitis. Values are mean ± range. (AN = acinar necrosis, HEM = hemorrhage, INF = leucocyte infiltration, ED = edema).

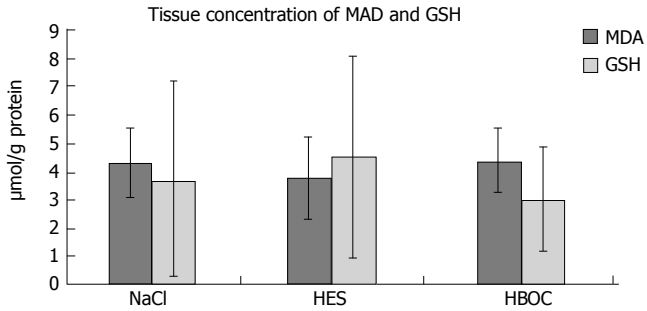

Figure 5 Tissue concentration of MDA and GSH in pancreatic tissue 6 h after induction of acute pancreatitis. Values are mean ± SD.

been performed since the late 1800 s. Over the years complications like renal toxic effects and short circulation time have been reduced. The new blood substitutes are able to replace blood, colloid and crystalloid solutions act as oxygen transporter in many experimental settings. The ability of potent oxygen transport to oxygen deprived tissue has been shown in different animal models[15,20].

Vincent *et al* investigated the influence of heme-binding proteins in heme-catalyzed oxidations. They confirmed previous studies that heme acts as a catalyst of H_2O_2-dependent lipid peroxidation[21]. It has been shown that diene conjugation caused by oxygen radicals gives an adequate assessment of lipid peroxidation in tissue extracts. Several studies emphasize the role of oxygen free radicals in the pathogenesis of experimental acute pancreatitis[1,3,22-26]. Oxygen radicals are generated by different biological systems. Sources are reperfusion injury as well as the respiratory burst of PMN-leucocytes[27-30]. Local tissue damage has successfully been treated by the use of radical scavengers[31,32]. However increased heme and oxygen availability like in HBOC-therapy might lead to higher generation of oxygen radicals and tissue damage caused by lipid peroxidation.

A single application of a hemoglobin based oxygen carrier in this model of severe acute pancreatitis reduces the tissue damage and ensures a stable microcirculation. Colloids like HES and Dextrane have also been proven to be an effective therapy for microcirculatory dysfunctions[33-36]. The superiority of HBOC[34,36,37] might be explained by the combining effect of a colloid and noncorpuscular oxygen carrier. HBOC releases oxygen in areas of poor perfundation and ensure tissue oxygenation.

However, the hypothesis that hyperoxygenation and additional oxygen supply might lead to an increased production of oxygen free radicals was not supported by this data. This study could not confirm former publications suggesting that increased heme availability promotes the enhencement of lipid peroxidation. Dunne *et al* demonstrated the reactivity of various modified hemoglobins to hydrogen peroxide in terms of free radical formation. They compared PHP hemoglobin (crosslinked between the β-subunits

and conjugated with polyoxyethylene) with DBBF hemoglobin (crosslinked between the β-subunits using dibromosalicylfumerate, and control HbA_0. All the blood substitutes generated free radicals. MDA- levels were equal in nearly all groups. When compared to sham operated animals MDA-levels in the HBOC groups reached the same levels. One explanation of this finding could be that the level of MDA and GSH might have reached their peak within the first hours of pancreatitis and decreased to normal levels after 6 h. This theory is underlined by former studies[23,32,38-39] Schoenberg *et al* determined the peroxidation products, conjugated dienes and MDA in hemorrhagic pancreatitis induced by retrograde injection of sodium-taurocholate . The tissue levels of MDA increased, reaching their highest level after 1 h. Two hours later the lipid peroxides had returned to control levels.

GSH-tissue levels also showed no statistically relevant differences in the groups. Compared to GSH-levels of sham operated rats a decrease was found which might be an indication for oxidative stress in all groups. Dabrowski *et al* were able to show a decrease in pancreatic GSH at an early stage of cerulein-induced pancreatitis in rats[40,41].

Other researchers were able to prevent local tissue damage in experimental pancreatitis by the use of radical scavenging therapy[26,31-32]. Since we could not detect differences in oxidative stress markers we would explain the reduced histopathological damage by an improved pancreatic microcirculation. An excess formation of free oxygen radicals in the setting of additional oxygen supply was not detected.

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RAPID COMMUNICATION

MRCP and MRI findings in 9 patients with autoimmune pancreatitis

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Abstract

AIM: To evaluate magnetic resonance cholangiopancreatography (MRCP) findings in conjunction with magnetic resonance (MR) images in autoimmune pancreatitis (AIP) patients.

METHODS: Nine patients with AIP underwent MRI, MRCP, endoscopic retrograde cholangiopancreatography (ERCP), computed tomography, and ultrasonography. The MRCP and MR images taken before and after steroid therapy were reviewed and compared with other imaging modalities. The MRCP findings of the AIP cases were compared to those of 10 cases with carcinoma of the head of the pancreas.

RESULTS: On MRCP, the narrowed portion of the main pancreatic duct noted on ERCP was not visualized, while the non-involved segments of the main pancreatic duct were visualized. The degree of upstream dilatation of the proximal main pancreatic duct was milder than that seen in cases of pancreatic carcinoma. Stenosis or obstruction of the lower bile duct was detected in 8 patients. MR images showed enlargement of the pancreas with decreased signal intensity on T1-weighted MR images, increased signal intensity on T2-weighted MR images, and, in 3 patients, a hypointense capsule-like rim. After steroid therapy, the previously not visualized portion of the main pancreatic duct was seen, along with improvement of the bile duct stenosis. Pancreatic enlargement decreased, and the abnormal signal intensity on both T1- and T2-weighted MR images became isointense.

CONCLUSION: MRCP cannot differentiate irregular narrowing of the main pancreatic duct seen with AIP from stenosis of the main pancreatic duct seen with pancreatic carcinoma. However, MRCP findings in conjunction with MR imaging of pancreatic enlargement that shows abnormal signal intensity on T1- and T2-weighted MR images are useful in supporting a diagnosis of AIP.

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Key words: Autoimmune pancreatitis; Magnetic resonance cholangiopancreatography; Magnetic resonance

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INTRODUCTION

Autoimmune pancreatitis (AIP) has recently been described as a peculiar type of chronic pancreatitis with underlying autoimmunity[1-5]. Patients with AIP almost always have enlargement of the pancreas[1-5], and their major presenting symptom (65%[6]-68%[7]) is obstructive jaundice due to obstruction of the bile duct. Since, pancreatic or bile duct carcinoma is suspected, pancreaticobiliary duct system is examined by endoscopic retrograde cholangiopancreatography (ERCP). The hallmark finding of AIP on direct pancreatogram is irregular narrowing of the main pancreatic duct; stenosis of the intra- or extrahepatic bile duct is frequently detected on cholangiography[1,2,5-7].

Magnetic resonance cholangiopancreatography (MRCP) has become popular as a non-invasive method for obtaining high quality images of the pancreaticobiliary tree[8]. Although the features of AIP on computed tomography (CT) and ERCP have been reported, the MRCP features of AIP have not been clarified. The aim of this study was to describe the MRCP findings in AIP patients before and after steroid therapy and to review the utility of MRCP for the diagnosis for AIP.

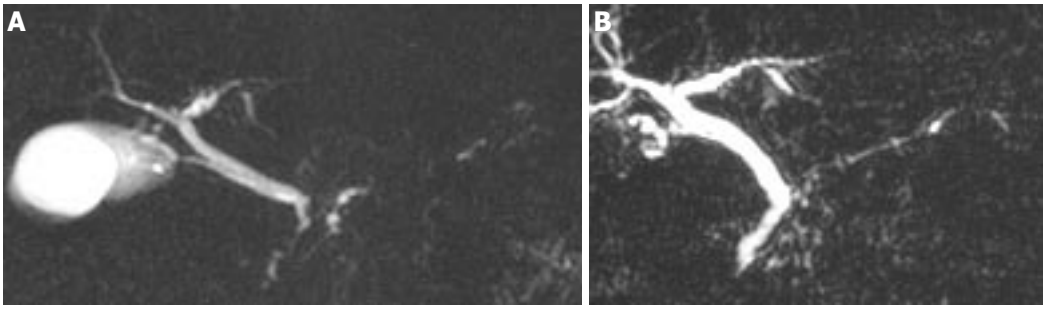

Figure 1 A: On MRCP of a patient with autoimmune pancreatitis, 2 segmentally skipped lesions of the main pancreatic duct in the head and body of the pancreas were not visualized; **B:** After steroid therapy, the entire MPD was seen on MRCP.

Table 1 ERCP and MRCP findings of patients with autoimmune pancreatitis

ERCP	MRCP
Diffuse narrowing of MPD in entire pancreas (n=1)	Non-visualized MPD in entire pancreas (n=1)
Segmental narrowing of MPD in the head of pancreas (n=3)	Non-visualized MPD in the head of pancreas (n=3)
Two skipped narrowing of MPD in the head and body of pancreas (n=3)	Two skipped non-visualized MPD lesions in the head and body of pancreas (n=3)
Segmental narrowing of MPD in the tail of pancreas (n=2)	Non-visualized MPD in the tail of pancreas (n=2)

MPD: main pancreatic duct.

MATERIALS AND METHODS

Subjects

Nine patients (6 males and 3 females, average age 60.3 years) with AIP who had MRCP in the time period from 1997 to 2005 were enrolled in this study. All patients had ERCP, ultrasonography (US), and CT. ERCP showed irregular narrowing of the main pancreatic duct in all patients [diffuse (n = 1), segmental-head (n = 3), segmentally skipped-head and body (n = 3), segmental-tail (n = 2)]. CT and US showed enlargement of the pancreas in 8 patients [diffuse (n = 3), segmental-head (n = 3), segmental-tail (n = 2)]. Six patients had elevations of serum IgG, 7 patients had elevations of serum IgG4, and 3 patients had antinuclear autoantibody. Six patients presented with obstructive jaundice. On cholangiography, stenosis of the bile duct was detected in 8 patients [lower common bile duct (n = 5) and intrahepatic and lower common bile duct (n = 3)]. All patients were given steroid treatment and responded both morphologically and serologically. From the radiological and serological findings, in addition to effectiveness of steroid treatment, all 9 patients were diagnosed as having AIP.

Methods

MRCP was done using a 1.5 T magnetic resonance imaging (MRI) machine (INTERA, PHILIPS Co Ltd, Netherlands) by coronal heavily T2-weighted single-shot rapid acquisition with relaxation enhancement. The MRCP and MR images taken before and after steroid therapy were reviewed and compared with other imaging modalities. The MRCP findings of the AIP cases were compared to those of 10 cases with carcinoma of the head of the pancreas.

RESULTS

The narrowed portion of the main pancreatic duct seen on ERCP was not visualized on MRCP, while the non-involved segments of the main pancreatic duct were seen on MRCP. On MRCP, the entire main pancreatic duct was not seen in 1 patient in whom the main pancreatic duct showed diffuse narrowing on ERCP. In 3 patients in whom the main pancreatic duct was segmentally narrowed in the head region on ERCP, the MRCP visualized the main pancreatic duct only in the body and tail of the pancreas, but not in the head region. In 2 patients, MRCP showed the main pancreatic duct only in the head and body of the pancreas, but not in the tail region. On MRCP, 2 segmentally skipped lesions of the main pancreatic duct in the head and body of the pancreas were not visualized in 3 patients (Figure 1A and Table 1). Although upstream dilatation of the proximal main pancreatic duct was detected in 6 patients, the degree of dilatation was milder than that in cases of pancreatic carcinoma. Stenosis or obstruction of the lower bile duct was detected in 8 patients, of whom 3 patients also showed stenosis of the intrahepatic bile duct accompanied by dilatation of the proximal bile duct, similar to the findings on ERCP.

All patients showed enlargement of the pancreas with abnormal signal intensity on T1- and T2-weighted images, though the degree of abnormal intensity was different among the cases. On T1-weighted MR images, the signal intensity of the affected pancreatic lesion was decreased compared to the signal intensity in the liver (Figure 2A). T2-weighted MR images showed increased signal intensity in the pancreatic parenchyma compared to that of the liver (Figure 2B). A capsule-like rim, which was hypointense on both T1- and T2-weighted images, was detected in 3 patients.

After steroid therapy, the portion of the main pancreatic duct that was previously not visualized on MRCP was seen (Figure 1B), and an improvement of the upstream dilatation of the proximal main pancreatic duct was evident. Stenosis or obstruction of the lower or hilar bile duct was found to be improved. The enlarged pancreas became normal in size (n = 6) or atrophic (n =

Figure 2 A: On T1-weighted MR images of a patient with autoimmune pancreatitis, the signal intensity of the affected pancreatic lesion was slightly decreased compared to the signal intensity in the liver; **B:** T2-weighted MR images showed increased signal intensity in the pancreatic parenchyma than that of the liver with a capsule-like rim which was hypointense; **C:** After steroid therapy, the enlarged pancreas became atrophic on T1-weighted MR images.

3) after steroid therapy. In all patients on both T1- and T2-weighted MR images, the abnormal signal intensity improved to being isointense with that of the liver, and the capsule-like rim disappeared (Figure 2C).

DISCUSSION

Since Yoshida *et al*[9] first proposed the existence of AIP in 1995, many cases of AIP have been reported. This unique form of chronic pancreatitis appears to be a distinct disease entity. AIP occurs predominantly in elderly males and frequently presents as obstructive jaundice[1,2,5-7]. This presentation is also typical of pancreatic carcinoma. As most patients with AIP respond to oral steroid therapy, an accurate diagnosis of AIP is necessary to avoid unnecessary laparotomy or pancreatic resection. Recently, MRCP has become a viable alternative to diagnostic ERCP as a non-invasive method for obtaining high quality images of the pancreaticobiliary tree[8]. We compared MRCP findings and MR images before and after steroid therapy in AIP patients.

The characteristic radiological images of AIP include irregular narrowing of the main pancreatic duct on ERCP and enlargement of the pancreas on CT and US[1-3,5-7]. The irregular narrowing of the main pancreatic duct on direct pancreatography, which is usually longer than the stenosis of the main pancreatic duct detected in patients with pancreatic carcinoma, is one of the useful findings that differentiate AIP from pancreatic carcinoma[1,3,10]. In the present study, the narrowing of the main pancreatic duct on ERCP was not visualized on MRCP, while the other region of the main pancreatic duct was visualized. After steroid therapy, the previously unvisualized lesions of the main pancreatic duct were seen. Thus, non-visualization of the main pancreatic duct might suggest narrowing of the main pancreatic duct. MRCP cannot differentiate irregular narrowing of the main pancreatic duct from stenosis of the main pancreatic duct in pancreatic carcinoma; thus, ERCP is necessary to diagnose AIP. However, MRCP findings of skipped, non-visualized main pancreatic duct lesions, accompanied by a lesser degree of upstream main pancreatic duct dilatation than that seen in pancreatic carcinoma, might suggest the presence of AIP. The MRCP of AIP patients frequently showed stenosis of the intra- or extrahepatic bile duct. T1-weighted MR images of AIP patients showed enlargement of the pancreas with decreased signal intensity, while T2-weighted MR images showed increased signal intensity, sometimes associated with a hypointense capsule-like rim in the involved segments. In previous reports, a decreased signal intensity on T1-weighted MR images, reflecting fibrosis in the pancreatic parenchyma, has been reported. Irie *et al*.[11] noted the hypointense capsule-like rim in the involved segment on T2-weighted MR images; this appears to be a characteristic finding of AIP, and is thought to be related to an inflammatory process involving peripancreatic tissue.

There have been no previous reports dealing with the change in the intensity in the pancreas of patients with AIP after steroid therapy. In the present study, on both T1- and T2-weighted MR images, abnormal signal intensity of the pancreas improved to isointensity with that of the liver. The enlargement of the pancreas and the capsule-like rim disappeared. Thus, MRCP and MR images are useful for evaluating the effectiveness of steroid therapy.

In conclusion, MRCP cannot differentiate the irregular narrowing of the main pancreatic duct seen with AIP from the stenosis of the main pancreatic duct seen with pancreatic carcinoma. However, the MRCP findings of skipped, non-visualized main pancreatic duct lesions, accompanied by a lesser degree of upstream dilatation of the main pancreatic duct, might suggest the presence of AIP. These MRCP findings, in conjunction with MR images of pancreatic enlargement showing abnormal signal intensity on T1- and T2-weighted MR images, are useful in supporting a diagnosis of AIP.

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RAPID COMMUNICATION

Incidence and predictors of upper gastrointestinal bleeding in patients receiving low-dose aspirin for secondary prevention of cardiovascular events in patients with coronary artery disease

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Abstract

AIM: The use of low-dose aspirin to prevent cardiovascular disease events is well established. However, the incidence and predictors of upper gastrointestinal bleeding (UGIB) with its use are unknown. We studied prospectively the incidence and outcome of peptic ulceration in low-dose aspirin users.

METHODS: A total of 991 patients with coronary artery disease (CAD) on low-dose aspirin were prospectively followed-up for two years for the occurrence and clinical features of first hospitalized episode of UGIB.

RESULTS: UGIB had a bimodal presentation with 45% occurring within four months of aspirin initiation and had an overall prevalence of 1.5% per year. There was no UGIB-related death. Hypertension (*OR* = 4.6, 95%CI 1.5 - 14.7, *P* = 0.009), history of peptic ulceration (*OR* = 3.1, 95%CI 1.1 - 9.0, *P* = 0.039), tertiary education (*OR* = 3.08, 95%CI 1.1 - 9.0, *P* = 0.039) and higher lean body mass (*P* = 0.016) were independent factors associated with UGIB. Use of nitrate did not reduce UGIB.

CONCLUSION: The incidence of UGIB in patients with CAD on long-term low-dose aspirin is low, but is accompanied with significant morbidity. With prolonged use of aspirin, UGIB continues to be a problem for those with risk factors and especially in patients with a history of peptic ulcers, in which UGIB tends to occur early after aspirin therapy.

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Key words: Coronary artery disease; Aspirin; Gastrointestinal bleeding

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INTRODUCTION

Anti-platelet therapy with low-dose aspirin (75-325 mg) reduces the risk of vascular events in patients with established coronary artery disease (CAD)[1-3]. However, the use of aspirin, even at a low dose for secondary prevention of cardiovascular events, is associated with a small but significant increase in the risk of upper gastrointestinal bleeding (UGIB). In addition, previous studies have also reported several risk factors, such as the use of non-steroidal anti-inflammatory drugs (NSAIDs) and old age, implicated in the development of UGIB in patients taking aspirin[4-8]. However, the incidence of aspirin-related UGIB in patients with CAD for the secondary prevention of cardiovascular events and the time frame of UGIB occurrence are uncertain. In addition, effects of several emerging therapies, such as the use of dual antiplatelet agents, nitrate or ulcer-protecting drugs for the development of UGIB, have not been systematically evaluated.

In a cohort of patients with established CAD recruited into a Cardiac Rehabilitation Programme, we prospectively monitored the time of occurrence of UGIB in patients taking low-dose aspirin for the secondary prevention of

cardiovascular events over two years. We also sought to determine the possible predictive factors associated with UGIB.

MATERIALS AND METHODS

Study population

In this prospective study, we recruited all patients with established ischemic heart disease (myocardial infarction, angina pectoris, angiographic-proven coronary artery disease) referred to a Cardiac Rehabilitation Programme in Tung Wah Hospital (an University-affiliated hospital) between September 1997 and April 2002. All the patients were started on low-dose aspirin (75-300 mg) according to referring physicians' discretion. Apart from collecting data on demographics, other parameters related to cardiac rehabilitation like education level, functional class, left ventricular function, exercise capacity, body mass index, body fat content, body lean mass content, medications used were also recorded. A database was specially designed to collect the required information prospectively. Patients were followed-up once every 3-4 mo and the occurrence of UGIB was noted. Detailed information about each UGIB event was obtained from interpretation and review of hospital records.

Upper gastrointestinal bleeding

The occurrence of UGIB was defined as the clinical presentation of hematemesis or passage of melena or unexplained drop in hemoglobin of ⩾ 20 g/L. Upper gastrointestinal endoscopy with therapeutic intervention(s) to obtain haemostasis (using epinephrine injection or heater probe application, when needed) was performed in all these patients presented with UGIB. *Helicobacter pylori* (*H pylori*) status was determined using a 24-h rapid urease test on antral biopsy specimens and histological detection of *Helicobacter*-like organisms using the haematoxylin and eosin stain, and Giemsa stain on biopsies taken from the gastric antrum and/or body. The rapid urease test at 24 h has been validated in our hospital with a sensitivity and specificity of 99% and 100%, respectively[9]. In addition, the use of histological examination of these biopsy specimens, the *H pylori* status was correctly evaluated even in patients with active UGIB, with the understanding that rapid urease test would be rendered inaccurate at those situations.

Statistical analysis

Univariate analyses were performed, using Student's *t* test and Mann-Whitney *U* test for continuous variables and Chi-square test or Fisher's exact test for categorical variables to assess the risk factors associated with UGIB. A multiple logistic regression model was designed to determine the factors (age, gender, educational level, occupation, hypertension, diabetes mellitus, renal impairment, history of peptic ulcer diseases, lean body mass, body fat, obesity, New York Heart Association (NYHA) functional class, left ventricular function, use of dual anti-platelet agents/NSAID/ulcer protective drug) associated with UGIB. To find the best model, a backward elimination stepwise procedure was carried out such that the factor would be eliminated from the analysis if the corresponding *P* value was greater than 0.15. A two-tailed *P* value of 0.05 or less was considered statistically significant.

Table 1 Baseline characteristics of 991 patients with coronary artery disease started on low-dose aspirin (mean ± SD, *n*, %)

	No UGIB (*n* = 962)	UGIB (*n* = 29)	*P* values
Age (mean ± SD)	64 ± 11	67 ± 11	0.663
Sex (M/F)	693/268	23/6	0.683
Hypertension (%)	519 (54)	22 (76)	0.008
Diabetes mellitus (%)	342 (35.5)	10 (34.5)	0.906
Renal impairment (Cr > 200 umol/L) (%)	42 (4.4)	5 (17.2)	0.001
Serum creatinine	120 ± 93	158 ± 103	0.002
History of MI (%)	536 (56)	16 (55)	0.095
History of PU (%)	74 (7.7)	9 (31.0)	0.006
LVEF < 40% (%)	388 (40.3)	11 (37.9)	0.034
NYHA (III-IV) (%)	82 (8.5)	6 (20.7)	0.048
% Body fat	34 ± 7	30 ± 7	0.036
% Lean body mass	66 ± 8	70 ± 7	0.049
Education level (tertiary) (%)	107 (11.1)	5 (17.2)	0.05
Nitrate use (%)	540 (56.1)	21 (72.4)	0.09

Cr: creatinine; MI: myocardial infarction; PU: peptic ulcer; LVEF: left ventricular ejection fraction; NYHA: New York Heart Association functional class.

RESULTS

Characteristics of patients and incidence of upper gastrointestinal bleeding (Table 1)

Between September 1997 and April 2002, a total of 991 patients with established ischemic heart disease were enrolled into the study and followed-up prospectively for two years. Of these, 716 (72.3%) were male (mean age, 64.8 ± 11 years); 552 (55.7%) had history of myocardial infarction (MI); 541 (54.8%) were hypertensive; 352 (35.5%) had diabetic mellitus; and 546 (54.7%) patients were smokers. Nitrate use was found in 561 (57.1%) patients. One hundred and twelve patients (11.3%) had received tertiary education. Twenty-nine patients developed UGIB over a follow-up period of two years, giving the incidence of 1.5% per year. For those who had a bleeding event, 72% did not have new-onset ulcer pain; 52% required blood transfusion (mean, 3.3 units; range, 1-8 units); 62% with a drop in hemoglobin of greater than 3 g/dL; 24% of these patients required more than one therapeutic upper gastrointestinal endoscopies for haemostasis. Peptic ulceration was the main cause of UGIB in our series (gastric ulcer, 38%; duodenum ulcer, 52%); aspirin-related hemorrhagic gastritis did not occur in our cohort. *H pylori* positivity status was present in 50% of patients presented with UGIB.

Of those who had UGIB, 21% of all the events occurred in the first month and 45% occurred in the first 4 mo after initiation of low-dose aspirin (early bleeders) (Figure 1). The 'late bleeders' (55%) developed UGIB at a mean of 20.8 ± 5.3 mo after initiation of therapy, with 88% (14/16) of them bled at least once after one year of aspirin therapy. In addition, about a third of those

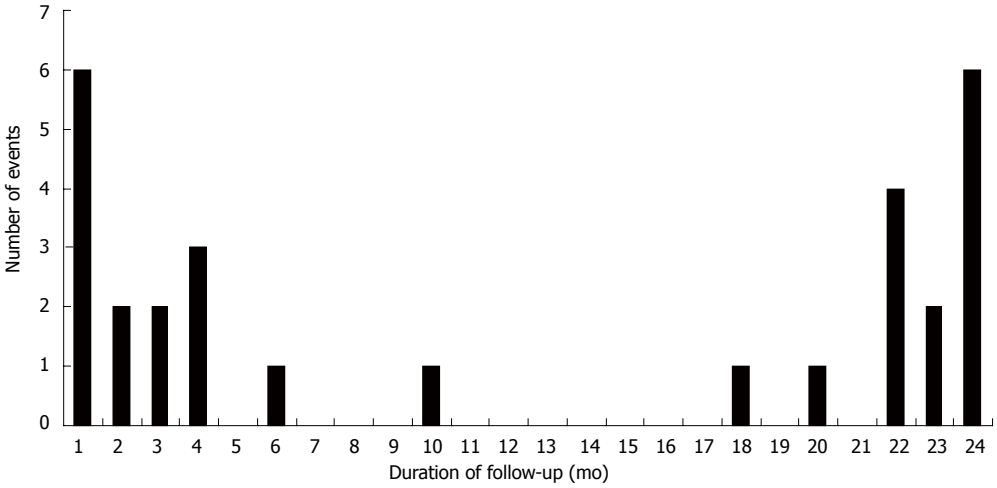

Figure 1 Bimodal presentation of upper gastrointestinal bleeding events in a two-year follow-up period.

who presented with UGIB (9/29) had a history of peptic ulcer/GIB/gastrectomy, in which 56% (5/9) of them were early bleeders. This indicates a 1:8 chance of having UGIB in patients with history of peptic ulceration while started on low-dose aspirin, which have a tendency to have early bleeding event; 97% of patients with UGIB required hospitalization. Death or myocardial infarction did not occur during the UGIB episodes and no patient required surgery for treatment of the UGIB. Nitrate use was recorded in 62% of the bleeders; 21% of those who had an UGIB were on ulcer-protecting drugs during presentation, but only 10% of them reported concurrent use of NSAID.

Predictors of UGIB

Univariate analyses showed that the presence of hypertension (*OR* = 3.1, 95%*CI* 1.3 - 7.7, *P* = 0.008), renal impairment (*OR* = 4.8, 95%*CI* 1.7 - 13.1, *P* = 0.001), history of peptic ulcer (*OR* = 4.6, 95%*CI* 2.0 - 10.7, *P* = 0.002), patients with impaired left ventricle function (EF < 40%) (*P* = 0.034) or NYHA functional class III-IV (*OR* = 3.0, 95%*CI* 1.2 - 7.6, *P* = 0.048), patients with high lean body mass (70% ± 7% *vs* 66% ± 8%, *P* = 0.049) or low body fat content (30% ± 7% *vs* 34% ± 7%, *P* = 0.036) and patients with tertiary education (*OR* = 3.1, 95%*CI* 1.1 - 9.0, *P* = 0.05) were predictors of the development of UGIB. Use of anti-ulcer therapy (in small number of patients) was not associated in prevention of UGIB. The use of nitrate did not reduce the risk of UGIB (*P* = 0.09). However, multivariate analyses using a logistic regression model demonstrated that only patients with hypertension (*OR* = 4.63, 95%*CI* 1.5 - 14.7, *P* = 0.009), history of peptic ulcer (*OR* = 3.1, 95%*CI* 1.1 - 9.0, *P* = 0.039), tertiary education (*OR* = 3.1, 95%CI 1.1 - 9.0, *P* = 0.039) or with high lean body mass (707% ± 7% *vs* 667% ± 8%, *P* = 0.0157) had a higher probability of UGIB while taking low-dose aspirin for secondary prevention of cardiovascular events (Table 2).

Table 2 Multivariate analysis of predictors of upper gastrointestinal bleeding in patients with coronary artery disease on low-dose aspirin (mean ± SD)

	95%*CI*	Odds ratio	*P* values
Education level (tertiary)	1.05 - 8.98	3.08	0.039
Hypertension	1.46 - 14.74	4.63	0.009
History of peptic ulcer	1.05 - 9.00	3.08	0.039
% Lean body mass (body fat content)			0.0157

DISCUSSION

To the best of our knowledge, this is the first prospective study for the first UGIB in patients started with low-dose aspirin for secondary prevention of cardiovascular events. UGIB occurred at a rate of 1.5% per year, and was due to peptic ulceration that required hospitalization and blood transfusion. Twenty-four percent of those with UGIB required more than one therapeutic endoscopy to secure haemostasis. On the other hand, new-onset ulcer syndrome was not present in majority of those patients that developed an UGIB. These data also showed that when patients started with low-dose aspirin and bled subsequently, 21% of them had the event in the first month of treatment, 45% of all bleeding events occurred in the first 4 mo of starting low-dose aspirin; whereas most of the other bleeding events took place after at least one year of therapy. This observation implies that not only the risk of UGIB associated with low-dose aspirin use does not decrease with time, rather, there exists a bimodal presentation of the event in our series with one peak occurring in the early phase of starting treatment and the second occurred beyond the first year. This is in contrary to previous studies, in which the rate of UGIB occurred in a constant fashion throughout the entire period of study[4,8]. There are several possible explanations for the difference. Unlike other studies, we prospectively recorded first episode of UGIB after aspirin initiation instead of recruiting those presenting with the event[5]. In addition, others reported the use of a higher-dose of aspirin for the prevention of cardiovascular events[6]. Furthermore, some animal studies reported the possibility of adaptation of the gastric mucosa to aspirin therapy with time, and this might explain why patients begin to have less bleeding after receiving several mo of aspirin therapy[14].

We demonstrated the predictors of UGIB events for patients taking low-dose aspirin. Better-educated people

were more likely to experience UGIB (*OR* = 3.1, the odds of UGIB was about 3 times more among people who received tertiary education than those who received secondary education or less). One explanation is that better educated people might be more compliant to prescribed medications, and they generally have much higher pressure/stress at work, and hence have a higher incidence of UGIB[10-11]. Consistent to previous reports, patients with a past history of peptic ulcer problems or UGIB were still at high risk of developing UGIB despite the use of low-dose aspirin[4]. Furthermore, we showed that most of them bled early on initiation of aspirin and thus deserve treatment for ulcer prophylaxis. In our study, patients with hypertension had the highest odds ratio for developing UGIB, concur with the traditional association between hypertension and the development of peptic ulcer[15]. Another interesting finding was that patients with a higher lean body mass had a higher probability for having an UGIB. A recent report suggested that obese patients are more prone to the development of aspirin resistance[12], so whether non-obese patients with slender-build (and hence a higher lean body mass content) are more susceptible to aspirin effect, including its toxicity on the stomach, will need further evaluation. Unlike a previous report[4], the use of nitrate was not linked to a reduction of UGIB in our study (P = 0.09). This may be due to a purely statistical problem as a large majority (57.1%) of the CAD patients in our cohort were on nitrate, which might mask the protective effect, if any of the latter; alternatively, other confounding factors, such as hypertension, leaner subjects in our series, might negate the effect of nitrate. The presence of renal impairment or heart failure/left ventricular dysfunction was believed to be predictors of UGIB as reported in our series using an univariate analysis, but these factors dropped out after multivariate adjustment. This might be due to the small number of events recorded in the present cohort. Our patients were younger (mean, 65 years; range, 35-87 years) compared to those in the previous study. With this age range, age appears not to be a predictive factor for UGIB.

The incidence of UGIB event in patients with CAD started on low-dose aspirin was low, and if occurred, was associated with good clinical outcome though most patients required hospitalisation. Nevertheless, with better understanding of the pathogenesis of the former and, in particular, its relation to *H pylori* status, our group has reported a possible method for the prevention of recurrence of peptic ulcer complication from long-term low-dose aspirin use[12]. On the other hand, our study showed that certain high-risk group of patients, especially those with history of peptic ulceration who tend to have early UGIB, should be target for primary prevention of peptic ulceration development and continued surveillance needed as well for these patients while on low-dose aspirin.

The small number of patients in our cohort that were on regular NSAID (1.1%) might not be able to show its deleterious effect on patients taking low-dose aspirin. Likewise, there was insufficient number of patients taking ulcer-protecting agents 21% to show a statistically significant benefit for patients taking these medications for ulcer prevention. However, the low usage of NSAID and ulcer-protecting drugs allowed us to determine the natural history of UGIB secondary to low-dose aspirin in this patient cohort, in which future therapeutic strategy can be planned. Finally, we failed to obtain the *H pylori* status for all patients, Regardless of whether they had or had not had an event, from which the value of that might be much more elicited. In addition, serological assay for *H pylori* infection was not universally available at time of our study. Nevertheless, local study reported that 55% of the population was infected with *H pylori* though symptoms and clinical disease developed in only a minority of infected individuals during their lifetime[13].

In summary, the incidence of UGIB in patients with CAD on low-dose aspirin remains low at 1.5% per year. Patients with hypertension, history of peptic ulcer problem, low body fat content (high lean body mass) and those that had received tertiary education were at risk of having an UGIB event. There exists bimodal presentation of the UGIB event with the early bleeders presenting in the first few months of starting treatment and the late bleeders after at least one year of therapy. Patients who had an event were likely to be hospitalized and required blood transfusion.

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RAPID COMMUNICATION

Relationship between IFN-γ gene polymorphism and susceptibility to intrauterine HBV infection

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Abstract

AIM: To explore the susceptibility of children to intrauterine HBV infection by studying the relationship between IFN-γ gene polymorphism, including IFN-γ+874A/T single nucleotide polymorphism(SNP) and CA repeat microsatellite polymorphism and intrauterine HBV infection.

METHODS: A TaqMan fluorescence polymerase chain reaction in the IFN-γ+874A/T single nucleotide polymorphism was tested in the intrauterine HBV infection group(group I) and the normal immune children group(group II). Capillary electrophoresis was performed in the above two groups to assay the IFN-γ CA repeat microsatellite polymorphism.

RESULTS: Frequencies of AA, AT and TT genotypes were 67.4%, 19.6% and 13.0% in the intrauterine HBV infection group, and 45.2%, 30.1% and 24.7% in the normal immune children group, respectively. A significant difference was found in the frequency distribution of IFN-γ+874 genotype between the two groups (χ^2 = 5.102, P = 0.02389). In the intrauterine HBV infection group the AA genotype was more common than in the normal immune group. Frequency of IFN-γ+874A allele was 77.17% in the intrauterine HBV infection group, and 60.27% in the normal immune children group. In the intrauterine HBV infection group the IFN-γ+874A allele was more common than in normal immune group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 7.238, P = 0.02389, OR = 2.228, 95% CI = 1.244-3.992). $(CA_{12})^+/(CA_{12})^+$ of IFN-γ CA microsatellite polymorphism was 11.90% in the intrauterine HBV infection group and 26.47% in the normal immune children group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 5.64, P = 0.0176). Frequency of IFN-γ CA repeat was 25% in the intrauterine HBV infection group and 43.38% in the normal immune children group. The frequency of IFN-γ CA repeat was less in the intrauterine HBV infection group than in normal immune group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 7.548, P = 0.0060).

CONCLUSION: There is a relationship between IFN-γ+874A/T SNP and intrauterine HBV infection as well as between IFN-γ CA microsatellite polymorphism and intrauterine HBV infection. IFN-γ gene polymorphism might be important in determining individual's susceptibility to intrauterine HBV infection.

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Key words: Interferon-γ; Gene polymorphism; Hepatitis B virus; Intrauterine

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INTRODUCTION

IFN-γ+874A/T SNP within the first intron can increase or decrease the binding of a particular transcription factor and ultimately the export of cytokine product[1-2]. The registered DNA sequences of the human IFN-γ gene deposited in Genbank show the presence of a CA repeat element in the first intron, 875 bp downstream from the start of the first exon. There are seven CA repeats of variable lengths In a consecutive study, 12 CA repeats are confirmed its role as a marker of high IFN-γ production[2]. We have reported in a previous study that higher IFN-γ secretion than spontaneous secretion could be found in the intrauterine HBV infection group and the normal immune children group with HBsAg stimulation, but the increase in the intrauterine HBV infection group is strikingly lower than that in the normal immune children group[3]. IFN-γ production is positively associated with IFN-γmRNA expression[3]. In the present study, we studied the IFN-γ+874 SNP and CA repeat microsatellite polymorphism in the intrauterine HBV infection group and the normal immune children group in order to explore the association between IFN-γ gene polymorphisms and susceptibility to intrauterine HBV infection.

MATERIALS AND METHODS

Subjects

The subjects were selected from outpatients who were in our hepatitis B (HB) vaccine follow up clinics. These subjects whose mothers were HBV carriers were inoculated with HB vaccine or HB vaccine plus hepatitis B immunoglobulin (HBIg) and followed up for serum alanine transaminase (ALT) and HBV marker. Intrauterine HBV-infected children were defined based on their positive HBsAg and/or HBV-DNA at birth and lasting for six months (group I). Normal immune children were defined based on their negative HBV marker after birth and high HBsAb titers (group II). ALT of all subjects was normal.

TaqMan fluorescence polymerase chain reaction

Genomic DNA was extracted from peripheral blood using the kits supplied by Sangon Bioengineering Company (Shanghai, China). Polymorphisms at +874 were identified using the ABI Prism 7700 fluorescent automatic sequencer. PCR products were obtained after amplification in a 10 μL volume containing 1×Taqman buffer A, 200 μmmol/L(each) dATP, dCTP, dGTP, 400 μmmol/LdUTP, 3.5 mmol/L $MgCl_2$, 300 μmmol/Lβ-actin forward primer, 300 μmmol/L β-actin reverse primer, 200 μmmol/Lβ-actin probe, AmpliTaq Gold, 0.025 U/μL DNA polymerase, 0.01 U/μL AmpErase UNG, 900 nmmol/L(each) forward & reverse primers, 250 nmol/L TaqMan MGB probe, 20ng DNA template. The forward primer sequence is 5'-ACA TTC CAC AAT TGA TTT TAT TCT TAC AAC A-3'. The reverse primer sequence is 5'-ACG AGC TTT AAA AGA TAG TTC CAA ACA-3'. The TaqMan MGB probe sequences are as follows: Fam: AAA TCA AAT CtC ACA CAC ACA C; Vic: AAA TCA AAT CaC ACA CAC ACA C. Forty-five cycles of PCR amplification were performed at 50 ℃ for 2 min, at 95 ℃ for 10 min, at 95 ℃ for 30 s and at 60 ℃ for 30 s.

Capillary electrophoresis

DNA was amplified in a 7.5 μL reaction volume containing 0.75 μL of 10 × buffer, 0.75 μL 25 mmol/L $MgCl_2$, 0.75 μL 2.5 μmmol/LdNTP, 2.5 U/μL Taq Gold, 0.06 μL DNA polymerase, 1 μL (each) forward & reverse primers, 2 μL DNA template, 1.19 μL ddH_2O. The forward primer sequence is CTT CGT TGC TCA CTG GGA TT-6-FAM. The reverse primer sequence is GCA AAG CCA CCC CAC TAT AA. The microsatellite region in the first intron of the IFN-γ gene was amplified on a PTC-100 thermal cycler. Following an initial denaturation (12 min at 95 ℃), samples were subjected to 10 cycles of PCR amplification, for each cycle, the sample was amplified at 94 ℃ for 30 s, at 53 ℃ for 30 s, at 75 ℃ for 45 s, followed by 30 cycles at 89 ℃ for 30 s, at 53 ℃ for 30 s, at 75 ℃ for 45 s, and a final extension at 4 ℃. The IFN-γ CA repeat microsatellite polymorphisms were monitored by ABI PRISM 3700 capillary electrophoresis automatic analysis.

Statistical analysis

IFN-γ+874A/T allele frequencies and IFN-γ CA microsatellite frequencies were evaluated by gene count. Allelic frequency was calculated with the following equation: allelic frequency = $n/2N$ (n represents the number of alleles, and N represents the total number of subjects). Allelic frequencies were compared using the chi-square test. A P value less than 0.05 was considered statistically significant. The strength of an association was expressed as odds ratio (OR) with 95% confidence interval. STATA 6.0 software was used to determine the statistical significance.

Table 1 Clinical characteristics of patients and controls

Subjects	*n*	Sex		HBV marker of mother		HBIg injection in pregnancy	
		Male	Female	$HBsAg^+$	$HBsAg^+$ and $HBeAg^+$	Yes	No
Group I	46	26	20	15	31	10	30
Group II	73	41	32	25	48	16	47
χ^2		0.0015		0.0339		0.0020	
P		0.9695		0.8539		0.9640	

Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

Table 2 Frequencies of IFN-γ+874 genotype in patients and controls

Subjects	*n*	Number of subjects and percentage of IFN-γ+874 genotype (%)		
		AA	AT	TT
Group I	46	31 (67.4)	9 (19.6)	6 (13.0)
Group II	73	33 (45.2)	22 (30.1)	18 (24.7)
χ^2		5.102		
P		0.02389		

Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

RESULTS

General clinical characteristics

No significant difference was found in sex, HBeAg positivity of mother and HBIg injection to pregnant women between the two groups (Table 1).

IFN-γ+874A/T SNP

The distribution of IFN-γ+874A/T allele frequencies in two groups was in accordance with Hardy-Weinberg hereditary equilibrium law. Frequencies of AA, AT and TT genotypes were 67.4%, 19.6% and 13.0% in the intrauterine HBV infection group and 45.2%, 30.1% and 24.7% in the normal immune children group, respectively. A significant difference was found in the frequency distribution of IFN-γ+874 genotype between the two groups (χ^2 = 5.102, P = 0.02389). In the intrauterine HBV infection group, the AA genotype was more common than in normal immune group (Table 2). Frequency of IFN-γ+874A allele was 77.17% in the intrauterine HBV infection group and 60.27% in the normal immune children group. In the intrauterine HBV infection group, the IFN-γ+874A allele was more common than in normal immune group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 7.238, P = 0.02389, OR = 2.228, 95%CI1.244-3.992)(Table 3).

Table 3 +874T and +874A IFN-γ allele frequencies in patients and controls

	Allele (*n*)	IFN-γ+874A/T allele frequencies (%)		OR (95% confidence interval)	χ^2	*P*
		+874A	+874T			
Group I	46 (92)	71 (77.17)	21 (22.83)	3.228 (1.244-3.992)	7.238	0.0071
Group II	73 (146)	88 (60.27)	58 (39.73)			

Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

Table 4 IFN-γCA microsatellite morphisms in patients and controls

	n	Number and percentage of genotype of IFN-γCA (%)		
		$(CA_{12})+/(CA_{12})^+$	$(CA_{12})+/(CA_{12})^-$	$(CA_{12})^-/(CA_{12})^-$
Group I	42	5 (11.90)	11 (26.19)	26 (61.91)
Group II	68	18 (26.47)	23 (33.82)	27 (39.71)
χ^2			5.640	
P			0.0176	

Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

IFN-γ CA microsatellite polymorphism

$(CA_{12})^+/(CA_{12})^+$ of IFN-γ CA microsatellite polymorphism was 11.90% in the intrauterine HBV infection group and 26.47% in the normal immune children group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 5.64, *P* = 0.0176)(Table 4). Frequency of IFN-γ CA repeat was 25% in the intrauterine HBV infection group and 43.38% in the normal immune children group. In the intrauterine HBV infection group, the frequency of IFN-γ CA repeat was less than in normal immune group. A significant difference was found in the frequency distribution between the two groups (χ^2 = 7.548, *P* = 0.0060)(Table 5, Figure 1).

Table 5 IFN-γ CA repeat times in patients and control

IFN-γ CA repeat	Group 1 (84) % (*n*)	Group II (136) % (*n*)	χ^2	*P*
11	1.19 (1)	0.74 (1)	0.119	0.7302
12	25 (21)	43.38 (59)	7.548	0.0060[1]
13	13.10 (11)	8.82 (12)	1.008	0.3155
14	40.48 (34)	33.82 (46)	0.989	0.3201
15	13.10 (11)	7.35 (10)	1.974	0.1600
16	4.76 (4)	3.68 (5)	0.155	0.6936
17	2.38 (2)	2.21 (3)	0.007	0.9327

[1] Significant difference between patients and controls.
Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

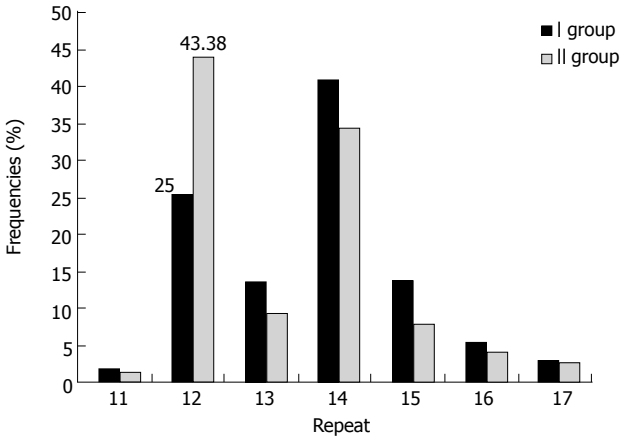

Figure 1 IFN-γ CA microsatellite polymorphisms in patients and controls. Group I: the intrauterine HBV infection group; Group II: the normal immune children group.

DISCUSSION

The clinical features of HBV infection depend on the activities of the host immune response and the virus. The cell-mediated immune response to HBV-encoded antigen is responsible both for viral clearance and for disease pathogenesis during this infection. T-helper lymphocyte response mediated through secretion of IFN-γ plays an important role in inducing viral clearance[4]. During the early aspecific phase of host defence, production of IFN-γ by natural killer(NK) cells plays an important role in bringing about acute inflammation. In the subsequent antigen-specific phase of the immune response, IFN-γ acts as a regulator of antigen presentation and proliferation as well as differentiation of lymphocytes. High-level production of IFN-γ during this phase plays an important role in viral clearance[5-6].

Cytokine production is genetically controlled. The cytokine genes SNPs in cis-acting regions can alter transcriptional activity and are associated with the production of cytokine[7]. Genetic susceptibility to diseases is likely influenced by common DNA variants in the form of SNPs[8-9]. Pravica *et al*[2] described a variable length CA repeat sequence in the first intron of the human IFN-γ gene and showed that 12 CA repeat is associated with high *in vitro* IFN-γ production. In a further study, a SNP T to A at the end of the CA repeat region in the first intron of the human IFN-γ gene(+874A/T) was described, showing an absolute correlation between the T allele and the high IFN-γ microsatellite polymorphism. The +874T allele is associated with the high production of genotype and the +874A allele is associated with the low production of genotype[10]. *In vitro* production of IFN-γ has a significant correlation with the CA microsatellite polymorphism. Twelve CA repeats have been shown to have a higher expression of IFN-γ[11-12]. Japanese researchers[13] have reported the frequencies of 13 CA repeats are significantly greater in patients with IgA nephropathy than in the healthy control group (43% versus 23%, *P*<0.05). High production of genotype (12 CA repeats) for IFN-γ may have an influence on acute rejection of kidney transplants[14]. The frequency of the IFN-γ12 CA repeats is significantly greater in WHO class V lupus nephritis patients than in WHO class IV patients[15]. Susceptibility to and severity of rheumatoid arthritis are related to a microsatellite polymorphism

within the first intron of the IFN-γ gene[16]. There is a highly significant increase in the low-production of IFN-γ genotype(13 CA repeats) in patients with type 1 diabetes compared with normal healthy controls[17]. This result suggests that polymorphisms of the IFN-γ gene may modify the function of this proinflammatory mediator and the response to pancreatic islet βcells. This +874A/T polymorphism coincides with a putative NF-Kappa B binding site, which might have functional consequences for transcription of human IFN-γ gene. Therefore, the T to A polymorphism might directly influence the level of IFN-γ production associated with the CA microsatellite polymorphism[18]. Patients with tuberculosis have a lower frequency of +874TT genotype than the controls, suggesting that genetically determined variability in IFN-γ and expression might be important for the development of tuberculosis[19]. A significant correlation has been detected between the presence of high-expression polymorphisms of the IFN-γ genes and bronchiolitis obliteration syndrome(BOS) after lung transplantation(P = 0.039). +874TT of the IFN-γ gene significantly increases the risk of BOS after lung transplantation[20]. There is no report on the relationship between IFN-γ gene polymorphism and susceptibility to intrauterine HBV infection.

In this study, the frequency of the +874AA genotype of the IFN-γ was greater in the intrauterine HBV infection group than in normal immune group, the $(CA_{12})^+/(CA_{12})^+$ of IFN-γ CA microsatellite polymorphism was 11.90% in the intrauterine HBV infection group and 26.47% in the normal immune children group. A significant difference was found in the frequency distribution between the two groups(χ^2 = 5.640, P = 0.0176). The frequency of IFN-γ CA repeat was less in the intrauterine HBV infection group than in the normal immune group. A significant difference was found in the frequency distribution between the two groups(χ^2 = 7.548, P = 0.0060). The findings suggest that the presence of low-expression polymorphism at +874 of the IFN-γ gene significantly increases susceptibility to intrauterine HBV infection. IFN-γ gene polymorphism might be important in determining an individual's susceptibility to intrauterine HBV infection and might result in viral persistence.

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RAPID COMMUNICATION

Diverticulitis in the young patient-Is it different ?

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Abstract

AIM: To prospectively study the incidence and the natural history of acute diverticulitis in young patients.

METHODS: A total of 207 patients hospitalized at our hospital between January 2000 to February 2005 with the diagnosis of acute diverticulitis were included. Their demographic characteristics, medical history, physical, radiographic and endoscopic findings as well as therapy were recorded. Patients were followed every 6 mo for the first year and later annually.

RESULTS: The mean patients' age was 61 (range 27-92) years. Twenty- five patients (12%) were younger than 45 years. Acute diverticulitis was significantly more prevalent among male in the young age group as compared to the older age group (19/25, 76% *vs* 61/182, 33%, respectively, P = 0.0001). Complications occurred more often in the young age group; 32% *vs* 13%, (P = 0.002). During follow-up, 6 patients (28%) remained asymptomatic in the young age group as compared to 87 patients (55%) in the older age group (P = 0.024). As a result, sigmoidectomies were performed twice as often in the young age group (38% *vs* 13%, P = 0.002).

CONCLUSION: Diverticulitis in young patients has a male predominance, a more aggressive course with a higher rate of complications and a higher recurrence rate. An earlier surgical approach might be considered in young patients with acute diverticulitis.

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Key words: Diverticulitis; Young patient; Complications; Sigmoidectomy

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INTRODUCTION

Diverticular disease of the colon is a common disease in Western countries. The development of colonic diverticula is associated with a low fiber diet, which is common in the developed countries[1,2]. The incidence of diverticular disease rises with age, and by the age of 85 about 65% of the population would have diverticuli[3,4]. It is much less frequent in the younger age group and less than 10% of the population under the age of 40 years would have diverticuli[5] . Most of the patients will remain asymptomatic, and only 10%-25% will become inflamed and evolve into diverticulitis[4,6-9]. The clinical presentation of acute diverticulitis varies widely from mild inflammation to full-blown perforation and peritonitis. In most patients however, the disease is mild and responds to antibiotic therapy[10] .

In recent years, there has been conflicting information regarding the incidence and natural history of diverticulitis in young patients. While earlier studies found the incidence to range between 2%-7%[11-15] , more recent studies reported a higher incidence ranging between 18%-34%[16,17]. The natural course and outcome of diverticulitis in the young patient has also been a matter of debate. While earlier studies reported a more severe course with a higher rate of complications in young patients with diverticulitis[1,2,11-14,18], recent reports suggested a milder course, not different from that in the general population[10,16]. All those studies, however, were retrospective. We performed a prospective study in which all patients with acute diverticulitis were enrolled and followed for a period of up to 5 years. We compared the natural history and the course of acute diverticulitis in the young age group to that of the older population.

MATERIALS AND METHODS

Patients

All patients with acute diverticulitis who were hospitalized at the Chaim Sheba Medical Center between January 2000 and February 2005 were enrolled. In fact, our patients' population included all patients who were diagnosed with acute diverticulitis in the emergency room, since the general policy of our institution is to hospitalize these patients regardless the severity of the disease.

Methods

The diagnosis of acute diverticulitis was based on lower

abdominal pain (usually on the left side), fever and leukocytosis. All patients underwent abdominal CT and only those with characteristic findings on CT were included. CT criteria for acute diverticulitis included the presence of colonic diverticuli with thickening of the colonic wall at the site of the diverticuli and peri-colonic fat infiltration. In some of the patients extra colonic air or fluid was seen as well. Patients with a lesion suspicious of colonic cancer on CT scan were excluded. Demographic characteristics of all patients and their medical history, concomitant use of medications, physical, laboratory, radiographic and endoscopic findings and their therapy were recorded.

Patients were followed up during their hospitalization and later by regular phone calls every 6 mo for the first year and then annually. Follow-up was conducted by a specialized study coordinator who was unaware of the patients' course of disease. At their follow-up visits or phone calls, patients were asked about the occurrence of attacks of lower abdominal pain with or without fever, hospitalizations and operations. Based on their reports, patients were classified into 4 major categories: patients who had undergone sigmoidectomy following their discharge, patients who had another episode of acute diverticulitis which required hospitalization and antibiotic therapy, patients who had LLQ abdominal pain for more than twice a week and for at least one hour's duration and patients who had not experienced symptoms suggestive of diverticulitis.

Data was analyzed for the whole group of patients and then for the two subgroups based on their age (older than and younger than 45 years). The age of 45 years was chosen as the average between the different ages used in the literature to define the young age group with acute diverticulitis (40 *vs* 50 years).

Statistical analysis

Patients' demography and other characteristics were analyzed by the Student's *t*-test for continuous variables and χ^2- test (with Yates correction) or Fisher's exact test for categorical variables. Statistical analyses were conducted by using Statsoft software (Tulsa, OK, USA).

RESULTS

Two-hundred and seven patients with acute diverticulitis were hospitalized between January 2000 and February 2005. Eighty were males and 127 were females. Their age ranged between 27-92 (mean 62 ± 12) years. Twenty-five patients (12%) were younger than 45 years. Their demography and co-morbidities are shown in Table 1. There was a striking male predominance in the young age group with a male: female ratio of 3:1. The older age group showed a female predominance with a female: male ratio of 2:1. As expected, the associated co-morbidities were far more common in the older age group, though only hypertension and ischemic heart disease reached statistical significance. The younger age group had almost no co-morbidities. However, twice as many abdominal operations had been performed in the young age group; 16% appendectomy and 12% inguinal hernioplasty as compared to 8.8% and 6%, respectively.

Table 1 Demographic data and co-morbidities of study subjects

Age (yr)	< 45 *n* = 25 (%)	>45 *n* = 182 (%)	*P* value
Gender (M/F)	19/6	61/121	0.0001
Co-morbidities			
Hypertension	1(4)	57 (31)	0.004
Hypothyroidism	0(0)	19 (10)	0.185
COPD	0(0)	10 (5.5)	0.481
Diabetes mellitus	0(0)	19 (10)	0185
Ischemic heart disease	0(0)	36 (20)	0.030
Hyperlipidemia	0(0)	17 (9)	0.227
Cholelithiasis	1(4)	11 (6)	0.682
Post appendectomy	4(16)	16 (8.8)	0.253
Post inguinal hernioplasty	3(12)	11 (6)	0.266
Malignancy	0(0)	7 (3)	0.684
Crohn's disease	1(4)	1 (0.5)	0.100
Chronic renal failure	0(0)	4 (2.2)	0.979

COPD: chronic obstructive pulmonary disease.

Table 2 Patients' complications during hospitalization

Age (yr)	< 45 *n* = 25 (%)	>45 *n* = 182 (%)	*P* value
Peri-diverticular air on CT	5 (20)	15 (8)	0.063
Abdominal abscess	2 (8)	4 (2)	0.150
Free perforation	1 (4)	5 (3)	0.540
Death	0 (0)	2 (1)	0.598
Total complication rate	8 (32)	24 (13)	0.030

Clinical course during hospitalization

All patients were followed during hospitalization and their clinical outcome was recorded. One-hundred and seventy-five patients (85%) had a mild course with no complications. The other 32 patients had a more severe and complicated course (Table 2). The most common complication was peri-diverticular air on CT which resolved in all patients by keeping patients fasting and on IV fluids and antibiotics. Six patients (2.8%) developed an abdominal abscess and 3 required CT guided percutaneous drainage. The abscesses resolved in all patients with no need for further intervention. There were 6 patients (2.8%) who developed free perforation and all were operated on. Two patients (0.1%) died during hospitalization, one from sepsis and another from multi-organ failure. Both were older than 80 years and had several co-morbidities. Their deaths were related to the diverticulitis.

The majority of the complications were more common in the young age group (Table 2). Abdominal abscess (8% in the young group *vs* 2% in the old group, $P = 0.155$) and peri-diverticular air on CT (20% in the young *vs* 8% in the old, $P = 0.063$) were the most common. The presence

Tables 3 Patients' follow-up after discharge

Age (yr)	<45 *n* = 21(%)	>45 *n* = 159 (%)	*P* value
Operations following discharge	8 (38)	20 (13)	0.002
Recurrent diverticulitis (not operated)	2 (10)	20 (13)	0.688
Abdominal pain[1]	5 (24)	27 (17)	0.442
No further symptoms	6 (28)	87 (55)	0.024

[1]Abdominal pain- pain more than 2 times per week that lasts for at least one hour.

of free perforation, however, was almost equal in both groups; 4% in the young and 3% in the old age group. The overall complication rate was higher in the young age group ($P = 0.03$).

Follow-up

Patients were contacted by telephone every 6 mo for the first year and yearly thereafter. The range of follow up was between 6 and 60 mo, mean: 30 ± 14 mo. Four patients in the young age group (16%) and 23 patients in the old age group (13%) were lost to follow up. Three of the patients in the old age group (2%) died during the follow-up period, all of whom were older than 75 years and their deaths were not related to the diverticular disease.

Data on patients in whom complete follow up was achieved is shown in Table 3. Sigmoidectomy was significantly more often performed in the young age group (38% *vs* 13%, $P = 0.002$). Most of the patients in the older age group, unlike the younger one, were asymptomatic during their follow up (55% *vs* 28%, respectively, $P = 0.024$).

DISCUSSION

Our prospective study showed that patients younger than 45 years comprised 12% of all patients who presented to our hospital with acute diverticulitis. The younger age group was male predominant and tended to have a more severe course with a higher complication rate, mainly peri-diverticular air and intra-abdominal abscess. Their recurrence rate during follow-up was higher and as a result their rate of sigmoidectomy was higher also. In 1986, Freischlag *et al*[13] found that in young patients, the first onset of diverticulitis was often severe and required urgent operation. Other retrospective studies supported this impression[1,2,18] and described a high recurrence rate and a high complication rate in their young patients with diverticulitis[19,20]. It was therefore recommended that an elective operation should be performed following the first episode of diverticulitis[17,19,21]. This policy was even further supported by Chautems *et al* who followed patients with acute diverticulitis under the age of 50 years for nearly 10 years. They found that in the young age group patients experienced a more aggressive disease[22]. However, other studies published in the last decade, failed to support this finding[10,16,23-26]. These studies reported a course of disease in young patients not different from the older age group and with a similar rate of complications. The conclusion of those recent studies was that elective surgery after one episode of acute diverticulitis in young patients is not advocated and such patients should be managed in a similar way to the older ones[25,26]. The explanation for the difference between studies that either support or reject a severe course in young patients is not clear. A possible explanation might be the difference in the study populations. Studies that found a similar course in both age groups had in their young age group a high proportion of Latino originated patients[10,16,23]. Young Latinos were reported to have an increased tendency to develop acute diverticulitis[27]. On the other hand, studies that described a severe course in young patients with acute diverticulitis did not include the Latino originated population. In fact, some of the studies were performed in Europe where Latino origin is quite rare[19,20,22]. Our study population also did not have patients of Latino origin. Thus, we speculate that young Latino patients with acute diverticulitis have a more benign course similar to that of older patients. The reason for this is not clear.

As a group, our young patients were more prone to recurrent symptoms. Our data is consistent with earlier studies that reported a higher recurrence rate of diverticulitis among young patients[2,20].The total complication rate was significantly higher in the young age group. None of the specific complications (free perforation, abdominal abscess or peri-diverticular air) reached statistical significance, probably because of the relative small number of patients in the young age group. Death occurred only in the older age group probably as a result of their higher co-morbidity rates.

In our study, the young patients with acute diverticulitis underwent significantly more sigmoidectomies as compared to our older patients. All those who underwent a sigmoidectomy had recurrent episodes of diverticulitis. The high rate of sigmoidectomies in the young age group was, therefore, the result of a more severe course of the disease in patients who were otherwise in good general condition.

Of interest is that the young age group underwent twice as many appendectomies and inguinal hernioplasties as compared to the older age group. Acute appendicitis in the young age group was even more prevalent than the life time risk for acute appendicitis in the general population (estimated 8.6% in men and 6.7% in women)[28]. The significance, if at all, of this observation is unclear. Misdiagnosis of previous attacks of acute diverticulitis is a possible explanation.

In conclusion, our prospective, long-term follow-up study shows that acute diverticulitis in general is a mild disease with a low complication rate. In young patients there is male predominance and the disease tends to be more aggressive with a high recurrence rate. An earlier surgical approach might be considered in young patients with acute diverticulitis.

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RAPID COMMUNICATION

Effects of ischemic preconditioning on cyclinD1 expression during early ischemic reperfusion in rats

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Abstract

AIM: To observe the effect of ischemic preconditioning on cyclinD1 expression in rat liver cells during early ischemic reperfusion.

METHODS: Fifty-four SD rats were randomly divided into ischemic preconditioning group (IP), ischemia/reperfusion group (IR) and sham operation group (SO). The IP and IR groups were further divided into four sub-groups (n = 6). Sham operation group (SO) served as the control group (n = 6). A model of partial liver ischemia/reperfusion was used, in which rats were subjected to liver ischemia for 60 min prior to reperfusion. The animals in the IP group underwent ischemic preconditioning twice for 5 min each time prior to the ischemia/reperfusion challenge. After 0, 1, 2, and 4 h of reperfusion, serum and liver tissue in each group were collected to detect the level of serum ALT, liver histopathology and expression of cyclinD1 mRNA and protein. Flow cytometry was used to detect cell cycle as the quantity indicator of cell regeneration.

RESULTS: Compared with IR group, IP group showed a significantly lower ALT level in 1 h to 4 h sub-groups (P < 0.05). Proliferation index(PI) indicated by the S-phase and G_2/M-phase ratio [(S+G_2/M)/(G_0/G_1+S+G_2/M)] was significantly increased in IP group at 0 and 1 h (26.44 ± 7.60% *vs* 18.56 ± 6.40%,41.87 ± 7.27% *vs* 20.25 ± 6.70%, P < 0.05). Meanwhile, cyclinD1 protein expression could be detected in IP group. But in IR group, cyclinD1 protein expression occurred 2 h after reperfusion. The expression of cyclinD1 mRNA increased significantly in IP group at 0 and 1 h (0.568 ± 0.112 *vs* 0.274 ± 0.069, 0.762 ± 0.164 *vs* 0.348 ± 0.093, P < 0.05).

CONCLUSION: Ischemic preconditioning can protect liver cells against ischemia/reperfusion injury, which may be related to cell proliferation and expression of cyclinD1 during early ischemic reperfusion.

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Key words: Liver; Ischemic preconditioning; CyclinD1; Proliferation

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INTRODUCTION

Hepatic ischemia/reperfusion (IR) injury is an important clinical problem occurring in hepatic resection or liver transplantation. Ischemic preconditioning (IP) has been proven to be an effective method protecting the liver against ischemia/reperfusion injury[1-3]. Many mechanisms are associated with this phenomenon[4-8], but some are still unclear. Previous studies showed that the analogous conditioning phenomenon induced by carbon tetrachloride (CCl_4) is related to hepatocellular proliferation[9]. Whether IP promotes hepatocellular regeneration remains unknown. Changes in cell proliferation can be determined by cell cycle analysis. S-phase and G_2/M-phase ratio is an indication the proliferation index (PI). Furthermore, cyclin and cyclin-dependent kinases (CDKs) are related to cell cycle regulation[10]. CyclinD1 is an important cyclin, which controls liver cell proliferation[11]. This study was to assess the effects of IP on cell regeneration and expression of cyclinD1 and to explore the possible mechanisms of IP protection.

MATERIALS AND METHODS

Experimental animals and grouping

Fifty-four male SD rats weighing 200-250 g, were randomly divided into ischemia group (IR group), ischemia preconditioning group (IP group) and sham operation group (SO). In IP group, each rat was subjected to 5 min

ischemia, followed by 5 min reperfusion, twice, prior to 1 h hepatic ischemia. Specimens were taken after 0, 1, 2, and 4 h of reperfusion, respectively (n = 6). Before the experiment, all rats were fasted for 12 h and housed in a 12 h dark/12 h light cycle.

Experimental animal model

A 70% partial liver ischemia/reperfusion model was established according to the method reported by Ohmori *et al*[12]. After anesthesia with ketamine (40 mg/kg), the belly of each rat was cut open from the center. The portal vein and hepatic artery supplying the left and median lateral hepatic lobes were dissected and clamped with a small artery clamp. After 1 h ischemia, blood flow was restored by unclamping the vessels. After 0, 1, 2, and 4 h of reperfusion, serum and liver tissue of each group were collected to assess the level of serum ALT, liver histopathology, expression of cyclinD$_1$ mRNA and protein. Liver tissue samples were homogenized and washed with PBS. The solution was filtered and centrifuged at 1000 r/min for 5 min. The cell pellet was resuspended in ice-cold ethanol to a final concentration of 80%. Flow cytometer was used to detect liver cell S-phase and G$_2$/M-phase ratio as the quantity indicator of cell regeneration. In IP group, each rat was subjected to 5 min ischemia followed by 5 min reperfusion, twice, prior to 1 h hepatic ischemia. The other procedure was the same as in IR group. In SO group, each rat was only cut open from the center without any other procedure.

Histological examination of liver

Liver tissue specimens were procured from the left lateral hepatic lobe, fixed with 10% neutral formaldehyde solution and embedded with paraffin. Sections were made and stained with H&E and observed under microscope.

Measurement of blood concentrations of ALT

Three mL of blood was withdrawn from the vena cava at certain time points, coagulated at room temperature, then centrifuged at 3000 r/min for 5 min for detecting the levels of alanine transaminase (ALT) with an automatic biochemistry analyzer (AU800, Olympus, Japan).

Detection of cell cycle

Single cell suspensions of hepatocytes were washed three times in PBS. RNase (1% w/v) was then added for 30 min at 37 ℃ and 50 mg/L PI (Sigma Company, Danmark) for 20 min. Samples were detected with a FACSORT flow cytometer (Becton Dickinson Company, USA), 10 000 cells were obtained by Cell Quest software and cell cycle was analyzed by Modfit software.

CyclinD$_1$ mRNA detection by reverse transcription-polymerase chain reaction (RT-PCR)

Primers synthesized according to the GenBank of Bioya Biotechnology Limited Company(Shanghai, China) included cyclinD1 sense primer (5' -TGGAGCCCCTGA AGAAGAG) cyclinD1 anti-sense primer (5'- AAGTGCGTTGTGCGGTAGC -3') amplified products (424bp), GAPDH sense primer (5'-GGCTGAGAATGGGAAGCTG-GTCAT-3') and GAPDH anti-sense primer (5'-CAGCCTTCTCCATGGTGGTGAA- GA-3) amplified products (152bp).

Total RNA was extracted from the frozen liver specimens with Trizol (GibcoBrl Company, USA). Reverse transcription synthesis of cDNA was carried out according to the instructions of SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen Company, USA). PCR was performed with a Gene Amp PCR System 2400 (PE Company, USA). The reaction system was composed of a cDNA template (1.0 μL), 2×buffer (12.5 μL), 5 mmol/L $MgCl_2$(2.5 μL), cyclin D1, GAPDH sense and anti-sense primer (0.5 μL respectively), SSRT polymerase (0.5 μL) and deionized water (6.5 μL). The reaction system was put into a PCR machine for amplification. Cyclin D1 and GAPDH were amplified for 35 cycles of PCR. The amplification conditions were at 55 ℃ for 30 min, at 94 ℃ for 2 min, at 94 ℃ for 30s, at 55 ℃ for 30 s, at 72 ℃ for 45 s, a final extension at 72 ℃ for 7min. The amplified products were preserved at 4 ℃. Ten μL of PCR products was electrophoresed on 1.5% agarose gel containing 5 μg/mL ethidium bromide (Sigma Company, USA). Marker was DL-2000 DNA marker. The results were detected by GeneSnap (SynGene, England) and analyzed by GeneTools (SynGene, England).

CyclinD$_1$ protein detection

Liver tissues were homogenized in 10 volumes of ice-cold buffer containing 50 mmol/L Tris–HCl (pH7.5), 150mmol/L NaCl, 1%Triton-X 100, 1 mmol/L EDTA,1mmol/L DTT,10μg/mL pepstatin,10μg/mL leupeptin, 10 μg/mL aprotinin, 1mM PMSF. The homogenates were centrifuged at 12 000 r/min for 15 min at 4 ℃. The supernatant was frozen and stored at -80 ℃ for further analysis. After measurement of the supernatant sample concentration, the sample was mixed with an equal volume buffer containing 0.1mol/LTris (pH 6.8), 100 mmol/L DTT, 2% SDS, 10% glycerin, 0.01% bromophenol blue and put in water bath to boil for 3-5 min. Fifty micrograms of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The stacking and resolving gels were composed of 4% and 10% polyacrylamide, respectively. After SDS-PAGE, the gels were electroblotted onto nitro-cellulose (NC) membranes at 100 mA for 2 h. The membranes were blocked for 1 h in blocking solution at room temperature, which contained 1 mL/L Tween 20 (PBS-T) and 50 g/L non-fat dry milk. Then they were immunolabeled with the primary antibody (1:500 rabbit anti-rat Santa Cruz Company, USA) in blocking solution for 1 h at room temperature and was washed three times with TBST for 15 min. The NC membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000 goat anti-rabbit Santa Cruz Company, USA) for 1 h at room temperature and washed three times with TBST for 15 min again. Color solution of diaminobenzidine (DAB) was prepared according to the manufacturer's directions (Maxim Company, China). After incubation with this color solution, the protein bands were visible on the NC membranes.

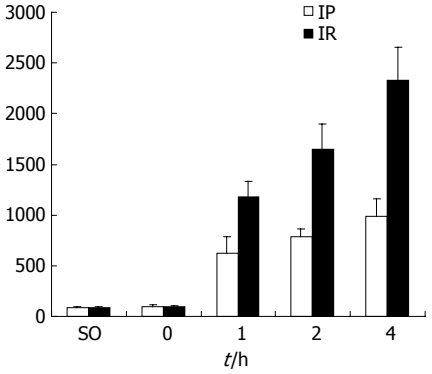

Figure 1 Serum ALT levels SO, IP and IR groups.

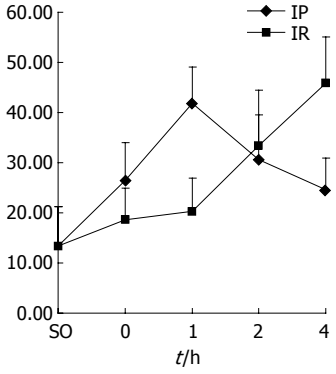

Figure 2 PI in SO, IP and IR groups.

Table 1 CyclinD1 mRNA relative activity in different groups (mean ± SD)

Group	IR	IP
SO	0.256 ± 0.042	0.256 ± 0.042
0 h	0.274 ± 0.069	0.568 ± 0.112[a,b]
1 h	0.348 ± 0.093	0.762 ± 0.164[a,b]
2 h	0.521 ± 0.157[b]	0.574 ± 0.172[b]
4 h	0.709 ± 0.178[b]	0.474 ± 0.144[a,b]

[a]$P < 0.05$ *vs* IR, [b]$P < 0.05$ *vs* SO.

Statistical analysis

The results were presented as mean ± SD. Multivariate analysis of variance and *t* test were performed by SPSS11.5 for window. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology

Swelling of hepatocytes and structural derangement were observed in IR group during the early period of reperfusion. Spot necrosis, hemorrhage and inflammatory cell infiltration could be seen in hepatic lobes or around central veins, with extended reperfusion time. Compared with IR group, IP group showed moderate hepatocyte swelling and less necrotic area.

ALT

The levels of serum ALT increased gradually as reperfusion time was extended in both groups. Compared with IR group, the level of serum ALT in IP group was significantly lower at all time points after reperfusion ($P < 0.05$) (Figure 1).

Hepatocellular proliferation

PI increased significantly at 0 and 1 h of reperfusion in IP group compared with IR group. There was a statistically significant difference ($P < 0.05$) (Figure 2).

Expression of cyclinD1 mRNA and protein

The RT-PCR results showed that cyclinD1 mRNA was expressed in SO, IR and IP groups. The expression of cyclinD1 mRNA in IP group increased at 0-1 h after reperfusion. Compared with IP group, the expression of cyclinD1 mRNA in IR group was significantly lower at 0-1 h after reperfusion ($P < 0.05$). No significant difference in the expression of cyclinD1 mRNA was found between SO and IR groups at 0-1 h after reperfusion ($P > 0.05$, Table 1, Figure 3A).

CyclinD1 protein was not detectable until 2 h after reperfusion in IR and SO groups. Whereas cyclinD1 protein was expressed in IP group soon after the reperfusion was begun (Figure 3B).

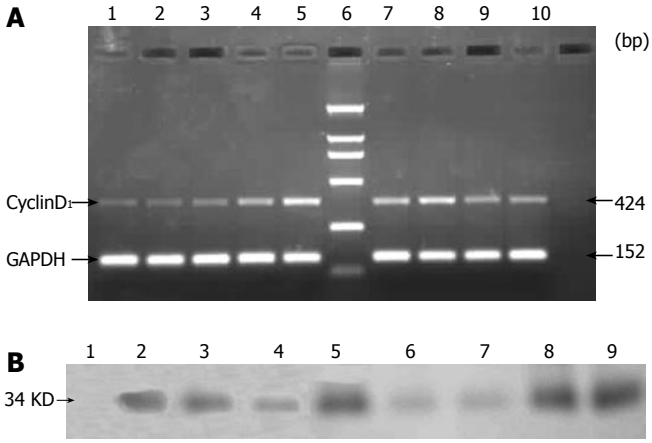

Figure 3 Expression of cyclin D1 mRNA (**A**) and cyclin D1 protein (**B**) at different time points in SO, IP and IR groups.

DISCUSSION

It is widely accepted that IP protects organs against IR injury. It has been reported that IR injury is related with many mechanisms, such as suppression of inflammatory reaction[13], improvement of local microcirculation[14], diminution of cell apoptosis and decreased production of local active oxygen-derived free radicals[15,16]. But some of the mechanisms remain uncertain. To probe into the mechanisms of IP protection may help to find an effective method against IR injury. In this study, we adopted the rat model of ischemic preconditioning against subsequent hepatic IR injury and confirmed that it could reduce the levels of serum ALT and the extent of liver necrosis.

Application of flow cytometry to cell cycle analysis makes it possible to analyze cell proliferation. Cell cycle can discriminate cells at the G_0/G_1-phase, S-phase and G_2/M-phase. It is considered that cells at the S-phase and G_2/M-phase can proliferate. S-phase and G_2/M-phase ratio could indicate the proliferation index (PI).The finding of the present work is that IP could promote liver cell proliferation during early ischemic reperfusion, which is possibly related to IP protection. Zhang *et al*[17] showed that proliferating liver cells can resist IR injury. The mechanisms of cellular proliferation involved in IP are still obscure, but

may be related to reactive oxygen species (ROS) and tumor necrosis facter-α (TNF-α).The liver underlying IP could generate ROS and TNF-α during early reperfusion[18,19]. ROS could stimulate cell mitosis and proliferation[20]. TNF-alpha is a known mitogen for hepatocytes and an important factor for liver cell proliferation. In the presence of anti-TGF-alpha neutralizing Abs, the mitogenic activity of TNF-alpha is abrogated[21].

Breakthrough in cell cycle regulation is the understanding of cyclin and cyclin-dependent kinase, which regulates cell cycle progression. Mitotic cellular division requires cells to leave the resting state (G_0/G_1) and proceed through the phase of DNA synthesis (S) and mitosis (G_2/M). There is a check point between G_0/G_1-phase and S-phase. CyclinD$_1$ is an important cyclin which can make cells pass through the check point and enter S-phase and G_2/M-phase. Higher expression of cyclinD$_1$ could promote cell proliferation in rat liver[11]. Our data showed that IP could stimulate cyclinD$_1$ mRNA and protein expression during the early reperfusion in IP group and not in IR group. The mechanisms may be related to the fact that IP activates the p38MAPK pathway[22], leading to activation of regulatory proteins and production of DNA synthetic proteins, such as cyclinD$_1$ and interleukin-6(IL-6)[23]. The p38MAPK pathway is an important signaling pathway involved in IP. Blockade of the p38MAPK pathway can lead to loss of protection of IP[24]. When the p38MAPK pathway is activated, the p38kinase transfers to cell nuclei and induces the production of cyclinD$_1$[25]. In our study, the cyclinD$_1$ protein was expressed in IR group 2 h after reperfusion, although cyclinD$_1$ mRNA was expressed during the early reperfusion. In SO group, cyclinD$_1$ mRNA was expressed but cyclinD$_1$ protein was not expressed. These data suggest that cyclinD$_1$ expression is posttranscriptionally regulated. Awad and Gruppuso[26] showed that a posttranscriptional mechanism regulating cyclin D1 content is involved in temporary hepatocyte growth arrest. They speculated that this posttranscriptional regulation may be downstream from the p38 mitogen-activated protein kinase pathway. The posttranscriptional regulation could keep adult hepatocytes in a quiescent state, but the mechanism is not completely clear.

In conclusion, IP could promote cyclinD$_1$ expression and lead to hepatocellular proliferation during early ischemic reperfusion, which is possibly one of the protective mechanisms against IR injury. However their underlying regulatory pathway and biological role remain unclear.

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RAPID COMMUNICATION

Lymph node micrometastasis and its correlation with MMP-2 expression in gastric carcinoma

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Abstract

AIM: To examine matrix metalloproteinase-2 (MMP-2) expression in gastric cancer tissues and to evaluate its relationship with lymph node micrometastasis.

METHODS: The authors studied 850 lymph nodes resected from 30 patients with gastric carcinoma who underwent gastrectomy with lymphadenectomy using reverse transcription polymerase chain reaction (RT-PCR) assay in addition to H-E staining. MMP-2 expression of the tumor tissues was detected by immunohistochemical technique (EliVision™ plus).

RESULTS: MMP-2 expression was positive in 21 (70%) cases and negative in 9 (30%) cases. No significant correlations were found between MMP-2 expression and other variables such as age, gender, tumor location, tumor diameter, Lauren classification and lymphatic invasion. In contrast, MMP-2 expression correlated significantly with depth of tumor infiltration (P = 0.022), lymph node metastasis (P = 0.030) and tumor differentiation (P = 0.043). Lymph node micrometastases were detected in 77 (12.5%) lymph nodes of 14 (46.7%) gastric carcinoma patients. MMP-2 expression was positive in 12 (85.7%) of the 14 patients with lymph node micrometastasis, and in 9 (56.3%) of the 16 patients without lymph node micrometastasis (P = 0.118).

CONCOUSION: Our results demonstrate that MMP-2 expression has significant correlation with tumor invasion, tumor differentiation and lymph node metastases. MMP-2 expression may be an important biological characteristics and significant prognostic parameter of gastric carcinoma. We also conclude that MMP-2 may participate in the development of lymph node micrometastasis of gastric carcinoma. Further investigations are needed to draw a conclusion.

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Key words: Gastric carcinoma; Lymph node micrometastasis; MMP-2; RT-PCR; Immunohistochemistry

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INTRODUCTION

Lymph node metastasis is the most important prognostic factor of gastric carcinoma. Many clinical studies demonstrate that the existence of lymph node micrometastasis is often overlooked by conventional histopathological examination (H-E staining)[1-10]. However, the mechanisms of lymph node micrometastasis remain unclear heretofore. MMP-2 has been reported to be associated with tumor invasion and lymph node metastasis of gastric carcinoma by mediating the degradation of extracellular matrix components[11-14]. However, the relationships between MMP-2 expression in cancer tissues and lymph node micrometastasis have not yet been explored. Therefore, the aim of this study was to investigate correlations between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma and possible relationships between lymph node micrometastasis and MMP-2 expression.

MATERIALS AND METHODS

Patients and specimens

A total of 850 lymph nodes resected from 30 patients with gastric carcinoma who underwent gastrectomy at the Department of Gastrointestinal and Pancreatic Surgery at Sun Yat-sen University of Medical Sciences were studied. There were 17 men and 13 women, ranging in age from 26 to 82 years, with a mean age of 56.8 years. None of these patients had received preoperative chemotherapy or radiotherapy. Total gastrectomy was performed in

16 patients, distal subtotal gastrectomy in 13 patients, and proximal subtotal gastrectomy in 1 patient. One patient underwent D1 lymphadenectomy, 22 patients underwent D2 lymphadenectomy, 4 patients underwent D3 lymphadenectomy, and 3 patients underwent palliative resection. According to the Lauren' criteria, 19 tumors were classified as diffusive type carcinomas, and 11 tumors were intestinal type carcinomas. Depth of tumor invasion and extent of lymph node metastasis were classified according to UICC TNM classification.

Half of each resected lymph node was fixed in 10% formalin and embedded in paraffin for routine histopathological examination. The other half was stored in 1ml RNA later (Sigma, USA) at 4 ℃ overnight, then transferred to a clean freezing tube and stored at -70 ℃ for RNA extraction. The resected primary tumors were also fixed in 10% formaldehyde and embedded in paraffin. Consecutive 4 μm sections were cut and stained with hematoxylin and eosin (H-E) staining and immunohistochenistry with anti-MMP-2 antibody.

RNA extraction

Lymph node samples were homogenized in 1 mL of Trizol Reagent (Invitrogen) per 50-100 mg of tissue using a power homogenizer. RNA extraction was carried out according to the protocol recommended by the manufacturer. Total RNA was dissolved in diethylpyrocarbonate-treated water and the volume and quality of the RNA then assessed by the ultraviolet spectrophotometer.

RT-PCR

Complementary DNA (cDNA) was synthesized and amplified from total RNA using the Access RT-PCR system (Promega). The primer sequences used for CK-20 detection were 5'-ggtcgcgactacagtgcatattaca-3' (sense) and 5' -cctcagcagccagtttagcattatc-3' (anti-sense)[15]. cDNA synthesis was monitored by beta-actin RT-PCR using the following primers: 5'- caaatgcttctaggcggact -3' (sense) and 5' - atgctatcacctcccctgtg -3' (anti-sense). The RT-PCR was performed in a 25 μL reaction mixture containing 11 μL nuclease-free water, 5 μL 5 × Reacton Buffer, 0.5 μL dNTP (10 mmol/L), 0.5 μL each of beta-actin primers (20 μmol/L), 1.25 μL each of CK-20 primers (10 μmol/L), 1 μL MgSO4 (25 mmol/L), 0.5 μL AMV reverse transcriptase (5 u/μL), 0.5 μL Tfl DNA polymerase (5 u/μL), and 3 μL RNA sample. The Access RT-PCR condition was set up as follows: 1 cycle at 48 ℃ for 45 min (reverse transcription), 1 cycle at 94 ℃ for 2 min (AMV RT inactivation), followed by 40 cycles at 94 ℃ for 30 s (denaturation) and at 62 ℃ for 1 min (annealing) and at 68 ℃ for 1.5 min (extension), followed by a final extension at 68 ℃ for 7 min. The resultant cDNA products of CK-20 and beta-actin were 121 base pairs and 381 base pairs, respectively. The RT-PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide.

In this report, "micrometastasis" in the regional lymph nodes was defined as metastasis that was detected only by the RT-PCR assay but not by routine H-E staining (Figure 1).

Immunohistochemistry

MMP-2 immunohistochemical staining was performed using EliVision™ plus IHC Kit (Maixin Biological, Fuzhou, China). After the sections were deparaffinized with xylene and dehydrated with ethanol, they were placed in 10 mmol/L citrate buffer and heated in a microwave at 700 W for 5 min for the retrieval of antigens in the specimens. Endogenous peroxidase activity was blocked by incubation of the slides in 3% hydrogen peroxide in absolute methanol at room temperature for 10 min. The sections were then incubated sequentially with 50 μL of mouse monoclonal antibody against MMP-2 (Maixin Biological, Fuzhou, China) overnight at 4 ℃, with 50 μL of polymer enhancer for 20 min, and 50 μL of polymerized HRP-anti mouse IgG for 30 min. The reaction products were visualized with diaminobenzidine (DAB Kit, Maixin Biological, Fuzhou, China), and sections were counterstained with hematoxylin, dehydrated, and evaluated under light microscope. Tris-buffered saline (TBS) solution was used instead of the primary antibody for negative controls.

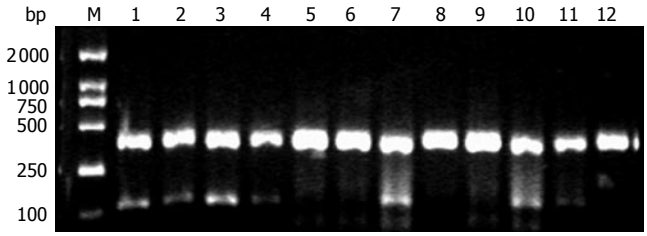

Figure1 Comparison of the results of RT-PCR and H-E staining. M: DL 2000 Marker; Lanes1-12: dissected lymph nodes. Lymph nodes of Lane 7, Lane 10, and Lane 11 were diagnosed lymph node micrometastasis.

For the purpose of data analysis, MMP-2 expression was graded according to the proportion of positive tumor cells. If more than 25% of the tumor cells were positively stained for MMP-2, the tumor was classified as positive MMP-2 expression. In contrast, if 25% or less of the tumor cells were positively stained, the tumor was classified as negative MMP-2 expression (Figure 2). The stained sections were observed independently by two pathologists who had no knowledge of the clinicopathological data.

Statistical analysis

Statistical analysis was performed by the Fisher's exact test to examine the association of MMP-2 expression of tumor tissues with the clinicopathologic characteristics of gastric carcinoma, and the relationship between MMP-2 expression of tumor tissues and lymph node micrometastasis was evaluated. Statistical significance was defined as $P < 0.05$.

RESULTS

Correlations between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma

MMP-2 expression was positive in 21 (70%) cases and negative in 9 (30%) cases. No significant correlations were found between MMP-2 expression and other variables such as age, gender, tumor location, tumor diameter, Lauren classification and lymphatic invasion. In contrast, MMP-2 expression correlated significantly with depth of tumor infiltration ($P = 0.022$), lymph node metastasis ($P = 0.03$) and tumor differentiation ($P = 0.043$) (Table 1).

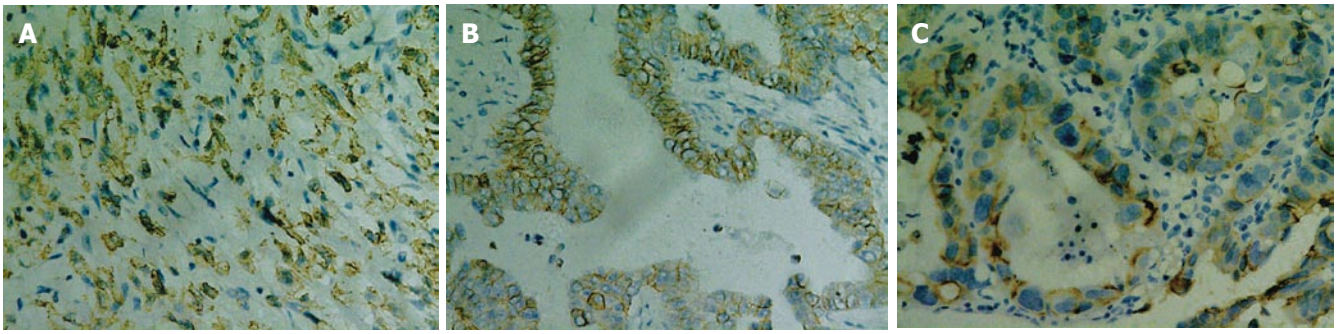

Figure 2 Expression of MMP-2 in gastric carcinoma. **A:** poorly-differentiated gastric carcinoma (× 400); **B:** moderately -differentiated gastric carcinoma (× 400); **C:** well-differentiated gastric carcinoma (× 400).

Table1 Correlation between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma

Variable	Patients (*n*)	MMP-2 expression		
		Negative (%)	Positive (%)	*P* value
Gender				
Male	17	6 (35.3)	11 (64.7)	
Female	13	3 (23.1)	10 (76.9)	*P* = 0.691
Age				
< 50 yr	10	1 (10)	9 (90)	
⩾ 50 yr	20	8 (40)	12 (60)	*P* = 0.204
Superficial diameter				
< 5 cm	17	7 (41.2)	10 (58.8)	
⩽ 5 cm	13	7 (15.4)	6 (84.6)	*P* = 0.229
Tumor location				
Upper/Middle third	17	5 (29.4)	12 (70.6)	
Lower third	13	4 (30.8)	9 (69.2)	*P* = 1.000
Histologic type				
Intestinal	11	5 (45.5)	6 (54.5)	
Diffuse	19	4 (21.1)	15 (78.9)	*P* = 0.225
Depth of invasion				
T1	5	4 (80)	1 (20)	
T2	16	4 (25)	12 (75)	
T3/T4	9	1 (11.1)	8 (88.9)	*P* = 0.022
Histologic differentiation				
Well	7	4 (57.1)	3 (42.9)	
Moderate	6	3 (50)	3 (50)	
Poorly	17	2 (11.8)	15 (88.2)	*P* = 0.043
Lymph node metastasis				
Positive	20	3 (15)	17 (85)	
Negative	10	6 (60)	4 (40)	*P* = 0.030
Lymphatic invasion				
Positive	18	3 (16.7)	15 (83.3)	
Negative	12	6 (50)	6 (50)	*P* = 0.102

Table 2 Correlation between MMP-2 expression of tumor tissues and lymph node micrometastasis

Micrometastasis	Patients (*n*)	MMP-2 expression		
		Negative (%)	Positive (%)	*P* value
Positive	4	2 (14.3)	12 (85.7)	
Negative	16	7 (43.7)	9 (56.3)	*P* = 0.118

Relationships between lymph node micrometastasis and MMP-2 expression

Lymph node micrometastases were detected in 77 (12.5%) lymph nodes of 14 (46.7%) gastric carcinoma patients. MMP-2 expression was positive in 12 (85.7%) of the 14 patients with lymph node micrometastasis, and in 9 (56.3%) of the 16 patients without lymph node micrometastasis. However, the difference between these two groups was not statistically significant ($P = 0.118$) (Table 2).

DISCUSSION

The protein MMP-2 (type IV collagenase) belongs to the family of metalloproteinases. Its function is related to cellular matrix degradation including basement membrane type IV collagen. MMP-2 has been reported to play an important role in the metastatic process and tumor invasion of gastric carcinoma[16-19].

Monig *et al*[13] investigated correlations between MMP-2 immunoreactivity and currently used classification systems and possible relationships between lymph node metastasis and MMP-2 expression in a prospective study. MMP-2 expression correlated significantly with depth of tumor infiltration, lymph node metastasis, distant metastasis, and UICC stage. In contrast, no significant correlations were found between MMP-2 expression and other variables such as age, tumor differentiation, WHO, Lauren, Goseki, and Ming classifications. Sundblad *et al*[12] also reported that MMP-2 expression showed significant correlations with parietal depth of infiltration ($P = 0.03$) and with metastases in regional lymph nodes ($P = 0.05$). However, Ko *et al*[20] reported that MMP-2 expression was not correlated significantly with lymph node micrometastasis or depth of invasion ($P > 0.05$). They also reported that TIMP-2 expression (MMP-2 inhibitor) showed a strong relationship with lymph node micrometastasis and depth of tumor infiltration ($P < 0.05$).

In the present study, MMP-2 expression was observed in 70% (21 of 30 cases) of gastric carcinoma. MMP-2 expression increased in accordance with the depth of tumor invasion. MMP-2 expression was more frequent in T3/T4 tumors (8 of 9 cases, 88.9%) and T2 tumors (12 of16 cases, 75%), as compared with T1 tumors (1 of 5 cases, 20%). The difference was statistically significant ($P = 0.022$). In 20 gastric carcinomas with lymph node metastasis 17 (85%) showed positive MMP-2 expression, while in 10 gastric carcinomas without lymph node metastasis only 4 showed positive MMP-2 expression ($P = 0.030$). In addition, we also found that there was a significant correlation between MMP-2 expression and tumor differentiation. 88.2% (15 of 17 cases) poorly differentiated gastric carcinomas showed MMP-2 expression, while positive MMP-2 expres-

sion of moderately- and well-differentiated gastric carcinomas were only 50% (3 of 6 cases) and 42.9% (3 of 7 cases) respectively. The difference was also statistically significant ($P = 0.043$).

Our results demonstrate that MMP-2 expression has significant correlation with tumor invasion, tumor differentiation and lymph node metastasis. For gastric carcinoma, MMP-2 expression may be an important biological characteristic and a significant prognostic parameter.

Recent advances in immunohistochemistry and molecular biology techniques have made it possible to detect lymph node micrometastasis not evidenced by routine H-E staining. 28%-68.1% of patients with gastric carcinoma were reportedly identified micrometastases in regional lymph nodes[21-25]. However, the mechanism of lymph node micrometastasis is still not completely known now. Therefore, the main objective of this study was to examine MMP-2 expression in the tumor tissues and explore its relationship with lymph node micrometastasis. Totally, 77 (12.5%) lymph nodes of 14 (46.7%) patients with gastric carcinoma were detected lymph node micrometastases. In 14 gastric carcinoma patients with lymph node micrometastases 12 (85.7%) showed positive MMP-2 expression, while in 16 gastric carcinoma patients without lymph node micrometastases only 9 (56.3%) were examined MMP-2 expression. The results indicate that MMP-2 may participate in the development of lymph node micrometastasis of gastric carcinoma, however, the difference was not statistically significant ($P = 0.118$). This may be explained by the fact that the cases in our study were comparatively few. To draw a further conclusion, a large-scale investigation is needed.

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RAPID COMMUNICATION

Clinical value of CT three-dimensional imaging in diagnosing gastrointestinal tract diseases

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Abstract

AIM: To discuss the clinical value of CT three-dimensional (3-D) imaging in diagnosing gastrointestinal tract diseases.

METHODS: Three-D imaging findings of 52 patients were retrospectively analyzed. Three-D imaging methods included shaded surface display (SSD), volume rendering (VR), virtual endoscopy (VE) and multiplanar reformatting (MPR). The diagnosis results of CT 3-D were evaluated by comparison with those of endoscopy and/or surgical finding.

RESULTS: Fifty-two patients with gastrointestinal tract diseases were diagnosed by CT 3-D imaging, of whom 50 cases were correctly diagnosed and 2 were misdiagnosed. There were 33 cases of gastric diseases (27 with carcinoma, 5 with peptic ulcer and 1 with leiomyoma) and 19 large intestinal diseases (10 with colon carcinoma, 2 with carcinoma of the rectum, 5 with colon polypus and 2 with tuberculosis of the ileocecal junction). Twenty-two cases with prominent lesions (9 with subsequent hollow lesions), 20 with stenosis of cavity (8 with concomitant prominent lesions) and 10 with hollow lesions (5 with concomitant prominent lesions) were shown in 3-D images. The minimal lesion shown was 1.0 cm × 0.8 cm × 0.5 cm.

CONCLUSION: CT 3-D imaging, a non-invasive examination without pain, can display clearly and directly the lesions of gastrointestinal tract with accurate location and high diagnosis accuracy. It is an important complementary technique to endoscopy.

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INTRODUCTION

Barium studies and endoscopy are two conventional examinations of gastrointestinal tract diseases. In recent years, with the advancement of spiral CT (single-detector row spiral CT, SDCT) and multi-detector row spiral CT (MDCT) scanner, CT three-dimensional (3-D) imaging has gained wide application and high diagnosis accuracy in gastrointestinal tract diseases[1-4]. Here we report the results of CT 3-D imaging in diagnosing gastrointestinal tract diseases in our hospital, and evaluate its clinical application.

MATERIALS AND METHODS

General information

From Jan 2000 to Oct 2005, 52 patents with complete clinical data were examined by 3-D imaging (From Aug 2001 to Oct 2003, due to short supply of effervescent granules, no such examination was carried out), among whom 30 cases were males and 22 females. The average age of the patients was 57.3 years (range, 20-85 years). Thirty-three patients were examined in stomach and 19 in large intestine.

Examination instrument

SDCT scanner (Somatom plus/4, Siemens, Germany) and workstation with 3-D imaging software (SUN magicview 1000) were used in 28 cases (18 in stomach and 10 in large intestine). MDCT scanner (LightSpeed/16, GE, America) and workstation with 3-D imaging software (Advantage Workstation 4.2) were used in 24 cases (15 in stomach and 9 in large intestine).

Examination methods

All the patients were required to fast for 12 h. The patient examined in the stomach took one pack (3 g) of efferves-

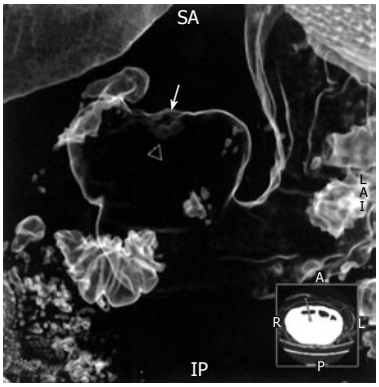

Figure 1 The ulcero carcinoma of stomach (on the anterosuperior wall of gastric antrum): the anterior-view of stomach on the VR image shows clearly the hollow lesion (↑) and the concomitant prominent lesion (△), with gas filling the stomach.

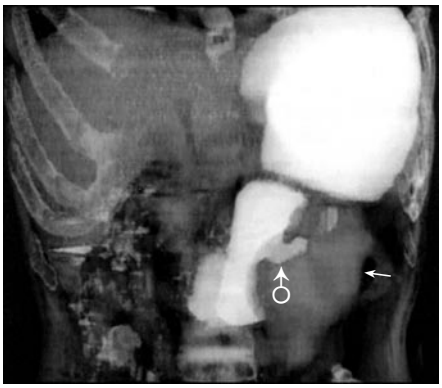

Figure 2 The ulcero carcinoma of stomach (on the greater curvature of stomach): the anterior-view of stomach on VR image shows the prominent lesion (↑) and the subsequent hollow lesion (♂), with 2% - 3% Omnipaque 300 filling the stomach.

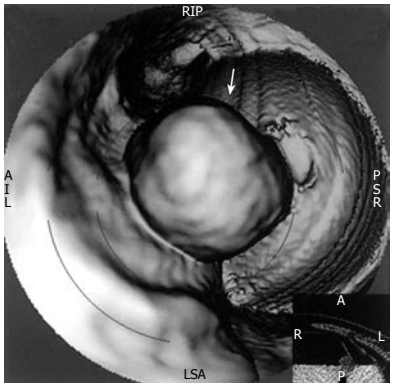

Figure 3 The leiomyoma of stomach (on the greater curvature of stomach): the axial-view of gastric cavity on VE image shows the prominent lesion (↑), with gas filling the stomach.

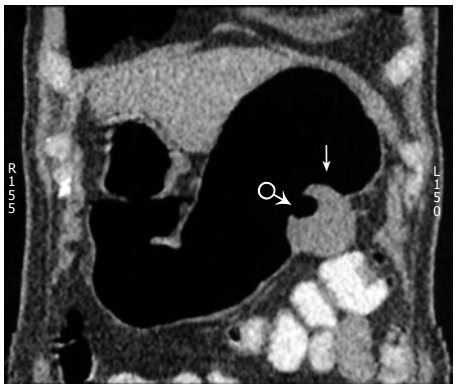

Figure 4 The same patient of **Figure 3**: the gastric coronal plane on MPR image shows the prominent lesion (↑) and the subsequent hollow lesion (♂).

cent granules (malu，Shanghai，China), lay in scanning bed and rolled to spread gas in stomach. A scout projection in supine position was made to confirm whether stomach was distended by gas. If distention was insufficient , another pack of effervescent granules (10 such cases in this study) or 500-1000 mL of 2%-3% Omnipaque 300 (Amersham, Shanghai，China) was added as an oral contrast agent to increase the contrasting effect and distending extent (8 such cases in this study). Another scout projection was scanned again. Holding breath for 10 s to 30 s, the patient was scanned with collimation of 5 mm (or 1.25 mm), pitch of 1.5 (or 1.375), increment of 2.4 mm (or 0.6 mm), scanning voltage of 120 kVp and a scanning current of 100-150 mA(the parameters in the brackets were used in scanning by MDCT). Another position of prone or lateral decubitus was often selected to better show some lesions (8 such cases in this study). The patients examined in the large intestines were asked to have a cleaning enema the night before and 30 min prior to the examination. A total of 600-1000 mL of air was infused through the anal canal as the contrasting agent. The following steps were the same as that used in stomach.

Image processing

Three-dimensional imaging methods included surface display (SSD), volume rendering (VR), virtual endoscopy (VE) and multiplanar reformatting (MPR). By selecting reconstruction thresholds of gastrointestinal walls from 15 Hu to 40 Hu and gastrointestinal cavities from -200 Hu to -1500 Hu, and necessary cutting technique, we obtained satisfactory 3-D images on the workstation. The exhibition of gastrointestinal tract lesions on the 3-D images was analyzed and measured, and the diagnosis results of CT 3-D imaging were evaluated by comparison with those of endoscopy and/or surgical finding.

RESULTS

Diseases diagnosed

Fifty-two patients with gastrointestinal tract diseases were histologically proven, of whom 33 cases had gastric diseases (27 with carcinoma, 5 with peptic ulcer and 1 with leiomyoma) and 19 had intestinal diseases (10 with colon carcinoma, 2 with carcinoma of rectum, 5 with colon polypus and 2 with tuberculosis of ileocecal junction). Fifty cases were correctly diagnosed (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6) and 2 misdiagnosed (1 with leiomyoma in stomach and 1 with tuberculosis in ascending colon).

Lesions shown

Prominent lesions (Figures 2-5) were shown in 3-D images in 22 cases (9 with subsequent hollow lesions, with maximal size of 5.4 cm × 6.0 cm × 2.1 cm and minimal of 1.5 cm × 1.2 cm × 0.8 cm), stenosis lesions (Figure 6) in 20 (8 with concomitant prominent lesions) and hollow lesions (Figure 1) in 10 (5 with concomitant prominent lesions, maximal of 2.0 cm × 1.8 cm × 1.1 cm, minimal of 1.0 cm × 0.8 cm × 0.5 cm). The locations, sizes and shapes of lesions in the 3-D images were close to that seen under endoscopy and/or in surgery.

Characteristics of imaging methods

Three-D images with VR, VE, SSD and MPR could

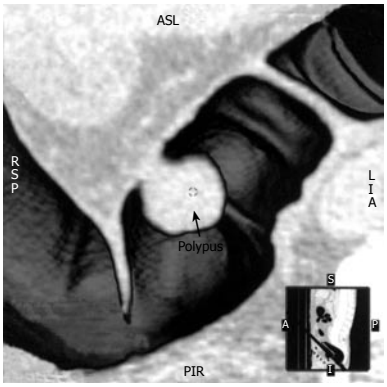

Figure 5 The polypus of colon: the anterior-view of colon on SSD image, by using the cutting technique, shows the prominent lesion (↑), with air filling the large intestine.

Figure 6 The carcinoma of descending colon: the anterior-view of large intestine on VR image shows the stenosis of intestinal cavity (↑), with air filling the large intestine.

show gastrointestinal tract lesions (Table 1), each with its own characteristics. SSD-imaging should be made with necessary cutting technique (Figure 5). VE-imaging could only show the extraluminal lesions. MPR-imaging had no perception of 3-D, but it could show clearly wall invasion (Figure 4). VR-imaging could show both extraluminal and intraluminal lesions (Figures 2, 6).

Table 1 Gastrointestinal tract lesions by3-D imaging with SSD, VR, VE and MPR

Methods	Prominence ($n=22$)			Hollow ($n=10$)			Stenosis ($n=20$)		
	G	M	B	G	M	B	G	M	B
SSD, cutting	18	4	0	7	2	1	19	1	0
V R	20	2	0	9	1	0	19	1	0
V E	20	2	0	6	3	1	17	3	0
MPR	18	4	0	4	4	2	18	2	0

G-good, indicates the sign was clear; M-medium, identifiable; B-bad, unclear.

DISCUSSION

Characteristics in showing lesions

Gastrointestinal tract lesions could be clearly shown by 3-D imaging, and the exact location and accurate size of a lesion could be obtained when it was more than 5 mm. In our study, the minimal lesion shown by 3-D image was 1.0 cm × 0.8 cm × 0.5 cm. This result is accordant with that reported by Zhang and Gluecker[3,5]. The main advantages of 3-D imaging over two-dimensional (2-D) multiplanar reformatting are added perspective and enhanced depth perception. The relationship between the lesion and adjacent structures can be better appreciated. It is often helpful to use the combinations of different 3-D imaging methods in different lesions, and to start with 2-D multiplanar reformatting and then to proceed to 3-D imaging[6]. Therefore in order to increase the diagnostic accuracy, we suggest several 3-D images be observed as well as the original axial images.

Influence of examination techniques on 3-D image

Theoretically, the quality of 3-D images is related to slice thickening, pitch and tube current[7]. Our study found that a satisfactory 3-D image of increased spatial resolution and decreased moving artificial shadows was obtained with thinner collimations and appropriate tube current and pitch. Interference of the contents in the gastrointestinal tract was avoided and adequate distention was obtained by using gas from effervescent granules of air as the contrasting agent of gastrointestinal tract, or by setting the patient in the prone, lateral decubitus and left posterior oblique position[8]. If gastrointestinal tract is not well distended or gastrointestinal wall has collapsed, some lesions may be overlooked and some similar disease may appear[9]. Traditionally, low or high attenuation contrasting agent can both be taken to enhance and distend the gastrointestinal tract. As to whether to take low or high attenuation contrasting agent, we think low-attenuation, e.g. gas, should be the first choice, as the agents are safer, well tolerated, and always result in good gastrointestinal tract distention. 2%-3% Omnipaque 300 may be optimal when stomach cannot be well distended. Horton *et al*[1,10] reported low-attenuation agents, e.g. oil-based oral contrast agents, whole milk and water, resulted in satisfactory evaluation of detection of subtle diseases of the gastric wall, and did not interfere with the manipulation of 3-D data sets. It is necessary to conduct further study on the clinical application of oral contrast agents.

Comparisons among 3-D imaging methods

All the 3-D imaging methods could show the lesions of gastrointestinal tracts, each with its own characteristics: MPR image could show the lesion in any section, e.g. sagittal or oblique. Though it could not get 3-D perception, it could remedy the defects of transverse-images, with the advantages of showing the gastrointestinal lesions of wall invasion. Images of VE and SSD with cutting techniques can show the size and shape of the lesions[6], especially intraluminal prominent lesions, and show the structure of gastrointestinal tracts, e.g. semilunar fold and ileocecal valve, from any directions without being influenced by a narrow cavity. They can better show the base and pedicel of lesions, but cannot discern surface coloration of lesion and extraluminal tumor invading [11]. VR images can be made translucent, similar to those of double contrast barium studies, and can demonstrate the overall contour changes of gastrointestinal tracts, but with poor mucosal information[12].

Clinical value of 3-D imaging

Accurate staging of gastrointestinal cancer is essential because surgical resection is the treatment only for local-

ized disease. Endoscopy and conventional barium study can only detect intraluminal lesions, but cannot evaluate extraluminal lesions. Traditional CT images can show anatomic structure and lesions of the gastrointestinal tract, but lack continuity and three dimensions. CT 3-D imaging has many advantages in the staging of primary tumor, assessing for local spread and detecting nodal involvement and distant metastases. It can provide valuable additional information and improve the detection and staging of both early and advanced gastric tumors and the detection of colorectal cancer and polypus[1,13,14]. CT 3-D imaging, with the same volume data obtained by spiral CT, especially by MDCT, can be repeatedly made with different reconstruction methods and avoid leakage of the section and moving artificial shadows. It has a high accuracy, sensitivity and specificity in showing prominent lesions and stenosis lesions, and in diagnosing gastric carcinoma and colon carcinoma[15]. 3-D imaging is a non-invasive examination without pain, and can easily be accepted by patients[14]. It may be used as an important complementary technique to endoscopy, particularly when the cavity is too narrow for endoscopy to go through. However, 3-D imaging examination has its limitations, e.g. it cannot show the color of lesions or make bioscopy[2].

Summary

CT 3-D imaging can clearly and directly show lesions of gastrointestinal tract. Diagnostic accuracy can be improved by observing different 3-D images together with original 2-D images. Three-D imaging is a non-invasive examination without pain, with characteristics of accurate localization of lesions and comprehensive observation of shapes. It is an important complementary technique to endoscopy.

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CASE REPORT

Successful aspiration and ethanol sclerosis of a large, symptomatic, simple liver cyst: Case presentation and review of the literature

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Abstract

Simple liver cysts are congenital with a prevalence of 2.5%-4.25%. Imaging, whether by US, CT or MRI, is accurate in distinguishing simple cysts from other etiologies, including parasitic, neoplastic, duct-related, and traumatic cysts. Symptomatic simple liver cysts are rare, and the true frequency of symptoms is not known. Symptomatic simple liver cysts are predominantly large (> 4 cm), right-sided, and more common in women and older patients. The vast majority of simple hepatic cysts require no treatment or follow-up, though large cysts (> 4 cm) may be followed initially with serial imaging to ensure stability. Attribution of symptoms to a large simple cyst should be undertaken with caution, after alternative diagnoses have been excluded. Aspiration may be performed to test whether symptoms are due to the cyst; however, cyst recurrence should be expected. Limited experience with both laparoscopic deroofing and aspiration, followed by instillation of a sclerosing agent has demonstrated promising results for the treatment of symptomatic cysts. Here, we describe a patient with a large, symptomatic, simple liver cyst who experienced complete resolution of symptoms following cyst drainage and alcohol ablation, and we present a comprehensive review of the literature.

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Key words: Simple hepatic cyst; Alcohol sclerosis; Laparoscopic deroofing

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INTRODUCTION

Liver cysts are classified as true or false, depending on the presence of an epithelial lining[1]. True cysts include congenital cysts (simple cysts and polycystic liver disease), parasitic (hydatid) cysts caused by *Echinococcus granulosis* and *multilocularis* tapeworms, neoplastic cysts (including cystadenoma, cystadenocarcinoma, cystic sarcoma, squamous cell carcinoma, and metastatic ovarian, pancreatic, colon, renal and neuroendocrine cancers), and biliary duct-related cysts (Caroli's disease, bile duct duplication, and peribiliary cysts)[1]. False cysts may be caused by spontaneous intrahepatic hemorrhage, post-traumatic hematoma, or intrahepatic biloma[1].

The pathogenesis of liver cysts is not clear. Simple liver cysts are congenital[2]. They are lined by cuboidal epithelium and originate from the abnormal development of intrahepatic ducts *in utero*. They are generally stable in size over time, but may slowly enlarge and occasionally become symptomatic due to mass effect, rupture, hemorrhage, or infection[3]. However, an enlarging cyst should prompt consideration of diagnosis other than simple cysts. Although simple cysts are generally solitary, more than one cyst may be present ("several solitary"), even in the absence of polycystic liver disease, as is the case with the patient described below.

We report herein a case of a patient with a large, symptomatic, simple hepatic cyst with resolution of symptoms immediately after therapy. In addition, we present a comprehensive literature review of diagnosis and treatment options of symptomatic hepatic cysts.

CASE REPORT

A 59-year-old African-American woman was referred to the Division of Gastroenterology at the University of Pennsylvania for evaluation of abdominal pain for 2 years. The patient described frequent, intermittent epigastric pain and bloating not associated with meals or bowel movements. Typically, pain would last for one-half hour before spontaneously resolving. She had twice presented to the emergency room with abdominal pain

Figure 1 Enhanced abdominal CT scan showing large, simple hepatic cyst.

Table 1 Rare complications of simple liver cysts

Obstructive jaundice
Infection
Intracystic haemorrhage
Spontaneous rupture
Inferior vena cava obstruction
Neoplastic transformation
Primary squamous cell carcinoma
Cystadenocarcinoma
Adenosquamous carcinoma
Adenocarcinoma
Hepatocellular carinoma
Cholangiocarcinoma

and was discharged without definitive diagnosis. She also complained of early satiety and occasional nausea without weight loss.

The patient was taking daily fiber supplements and had regular, daily bowel movements. Reflux symptoms were well controlled with esomeprazole. Hypertension was treated with metoprolol and amlodipine. Other chronic medications were progesterone and estrogen. She denied non-steroidal anti-inflammatory drug usage. Physical examination was unremarkable.

Laboratory evaluation showed normal liver-associated enzymes, metabolic panel, complete blood count, and urinalysis. Screening colonoscopy, performed two years prior to presentation, revealed internal hemorrhoids and melanosis. Upon presentation, she underwent double contrast upper gastrointestinal examination, which suggested antral gastritis and a hiatal hernia. On subsequent upper gastrointestinal endoscopy, the mucosa appeared normal, and a medium-sized hiatal hernia was present. Treatment with a proton pump inhibitor, fiber, and hyoscyamine failed to improve symptoms by six months.

The patient underwent abdominal CT scan with intravenous and oral contrast, which showed a large (7.7 cm) hepatic cyst in the lateral segment of the left lobe, as well as several other smaller cysts (Figure 1). Gallstones without gallbladder wall thickening or pericholecystic fluid were also visualized. On ultrasonography, tenderness was elicited specifically over the site of the cyst, which measured 10.3 cm in its longest dimension.

After several months of expectant management, the patient was referred to interventional radiology for drainage of the large hepatic cyst. Under ultrasonographic guidance, an 8-gauge French catheter was placed into the cyst, serous non-bilious fluid was aspirated, and the catheter was placed to gravity drainage. One week later, after drainage had ceased, the patient underwent ethanol sclerosis. At 4 mo follow-up, the patient was completely symptom-free. Follow-up ultrasound showed complete cyst resolution.

DISCUSSION

Previous studies, based on autopsy and surgical series, estimated a very low prevalence of simple non-parasitic hepatic cysts (0.14%-0.17%)[4]. More recently, among patients referred for abdominal ultrasonography, the prevalence of simple hepatic cysts has been reported 2.5%-4.65%[5,6]. Liver cysts have been recognized increasingly as the routine use of imaging studies becomes more widespread. Hepatic cysts may be more common in women[5] and in patients older than 40 years[6]. Symptoms, though quite rare, may be related to the space-occupying effect of large cysts[7] and may be more common in right-sided cysts[8]. Symptoms may include abdominal discomfort, chronic right upper quadrant or epigastric abdominal pain, early satiety, dyspnea, increasing abdominal girth, nausea, and vomiting[7,9]. Sanfelippo *et al*[4] reported that among 15 symptomatic patients with solitary non-parastic liver cysts, abdominal mass was present in 54%, hepatomegaly in 40%, abdominal pain in 33% and jaundice in 9% patients.

Although the natural history of simple hepatic cysts is not well known, complications appear to be quite rare (Table 1). Obstructive jaundice caused by solitary non-parasitic liver cyst is rare[11-15], and such cysts are usually large and located centrally in the liver, causing compression of the hepatic hilum[13]. However, sometimes even small hepatic cysts (3 cm in diameter) may cause common bile duct stenosis and intrahepatic biliary dilatation[15].

Liver cysts may also cause obstruction of the inferior vena cava, which may lead to massive edema of the legs[16,17] and scrotum[16]. Infections of simple hepatic cysts with *Klebsiella pneumoniae*[18] and *Escherichia coli*[19], presenting with acute onset of right upper quadrant abdominal pain, diarrhea, and fever have also been reported. Other documented complications include intracystic haemorrhage[19-23] and spontaneous rupture[23]. Neoplasms arising from solitary non-parasitic liver cysts, including primary squamous cell carcinoma[24-26], cystadenocarcinoma, adenosquamous carcinoma, adenocarcinoma, hepatocellular carinoma, and cholangio carcinoma[27-29], have been reported, but appear to be very rare. A possible association with Peutz-Jeghers syndrome has also been suggested[30].

Imaging modalities (ultrasound, CT, and MRI) are highly accurate for diagnosing simple cysts (Table 2). Large differences in echogenicity between hepatic parenchyma and cyst fluid allow for easy recognition of simple cysts by ultrasound[31]. The ultrasonographic appearance of simple cyst is characterized by well-defined, echo-free lesions with

Table 2 Radiologic features of simple hepatic cysts

Features supporting diagnosis of a simple cyst	Features not supporting diagnosis of a simple cyst
Anechoic lesion	Echoic lesion
Thin wall	Thick wall
Absence of septations	Presence of septations
No peripheral enhancement on CT/MRI	Peripheral enhancement on CT/MRI
Homogeneous appearance	Heterogeneity within the cyst
	Hydatid sand
	Presence of daughter cysts
	Heavy wall calcifications

Table 3 Alternative explanations for symptoms in patients with simple hepatic cysts

Diagnosis
Biliary colic
Gastroesophageal reflux
Peptic ulcer
Non-ulcer dyspepsia
Irritable bowel syndrome
Chronic pancreatitis
Abdominal wall pain syndrome

good through transmission and an imperceptible wall[3,32]. The presence of acoustic enhancement results from relative lack of absorption and reflection of sound by cyst fluid, as compared with hepatic parenchyma[32]. On CT scan, simple cysts appear as well-demarcated, water-density sacs, which do not demonstrate peripheral enhancement after intravenous contrast [3]. The presence of septations suggests that a cyst is not simple. Occasionally, large simple cysts may have "septations" due to hemorrhage. MRI shows simple cysts as hypointense lesions on T1-weighted and hyperintense on T2-weighted images[32,33]. Simple cysts differ from cavernous hemangiomas in that they are more hypointense on T1-weighted and of equal hyperintensity on T2-weighted images[33]. Radiologic characteristics which would argue against a cyst being simple include a thick wall, peripheral enhancement on CT or MRI, heterogeneity within the cyst, and an increase in size over time. Hepatic cysts should be differentiated from hepatic abscesses, hematomas, necrotic metastases, an intrahepatic gallbladder, biliary cystadenoma, and echinococcal (hydatid) cysts[32].

Radiologic imaging can accurately identify hydatid cysts, with an accuracy of 96% in one series[34]. Presence of hydatid sand, internal sepatations, daughter cysts, and heavy wall calcifications argue for *Echinococcus granulosis* infection, instead of a simple cyst. Epidemiologic features and serology in combination with radiologic imaging generally lead to the correct diagnosis non-invasively.

Distinction between cystic and solid hepatic lesions can be made accurately by ultrasonography, though CT or MR imaging may be more sensitive for the detection of focal hepatic masses[35]. The presence of any peripheral enhancement or thick-walled component suggests the possibility of hepatic abscess or neoplasm[32]. Small hepatic lesions with diameter < 1 cm are difficult to classify[36]. Such lesions should be differentiated from benign cysts, hepatic metastases with central necrosis, and microabscesses[36]. In order to differentiate small cysts from small metastases, follow-up imaging may be considered in selected cases[36].

The vast majority of simple hepatic cysts require no treatment. Large cysts (diameter of 4 cm or more) can be followed for stability with repeated imaging[37]. If the cyst remains unchanged for 2 years, further monitoring may be discontinued[37]. Symptomatic or enlarging cysts require consideration of alternative diagnoses, including cystadenoma, cystadenocarcinoma, and hepatic metastases. It should be stressed that attribution of symptoms to simple cysts should be undertaken with caution after excluding alternative diagnoses. Epigastric or right upper quadrant abdominal pain provoked by eating may indicate biliary colic, if gallstones are present. A successful trial of acid suppression therapy points to gastroesophageal reflux disease. Selected patients may undergo upper gastrointestinal endoscopy to diagnose erosive esophagitis or peptic ulcer disease. Esophageal pH monitoring can confirm the diagnosis of gastroesophageal reflux. If symptoms fluctuate in concert with changes in stool frequency or form, a diagnosis of irritable bowel syndrome should be suspected. Finally, a diagnosis of non-ulcer dyspepsia may be entertained in patients with unremarkable upper gastrointestinal endoscopy who have continued prominent upper abdominal pain, possibly in association with nausea and vomiting (Table 3). If the preceding diagnoses can be confidently excluded, then treatment of a large, symptomatic hepatic cyst may be undertaken. Treatment options include needle aspiration with or without injection of sclerosing solution, internal drainage with cystojejunostomy, wide deroofing, and different degrees of liver resection[37].

Percutaneous US- or CT-guided needle aspiration of hepatic cysts is associated with high recurrence rates (78%-100%)[38,39]. Several small case series have demonstrated efficacy for the performance of US- or CT-guided needle aspiration of hepatic cysts combined with alcohol injection[40-44]. Because US-guided aspiration with ethanol sclerosis is generally safe, effective, and relatively non-invasive, it may be a first-line treatment for selected symptomatic congenital hepatic cysts, especially in patients with high surgical risk or polycystic liver disease[15]. The 95%, 96% and 99% alcohol solutions are equally safe and effective[15,42,45]. Enough alcohol should be instilled to replace 25% of the aspirated cyst fluid volume[46]. For larger cysts (> 400 mL), multiple alcohol injections in the same sitting have been proposed[46]. Alcohol fixes the cells lining the cyst cavity, disabling their ability to secrete fluid and promote cyst enlargement[46]. Recurrence may occur if alcohol does not come in contact with all cells lining the cyst cavity[46] and may be more common in uncooperative patients[47]. Combinations of percutaneous aspiration with other sclerosing substances, such as iophendylate (pantopaque)[48], tetracycline chloride[49-52], doxycycline[52], minocycline chloride[53-56], and hypertonic saline solution[8] have also achieved good outcomes.

Several recent series have demonstrated good results for laparoscopic deroofing procedures. Widest possible excision of the cystic wall and concomitant argon beam

Table 4 Comparison of treatment options for symptomatic simple liver cysts

Treatment options	Advantages	Disadvantages
Observation alone	- Because most cysts are asymptomatic, intervention is unlikely to be helpful and may be harmful	- Only effective cyst treatment can prove whether symptoms are related to the cyst
US-guided aspiration	- Simple procedure - May be used as a diagnostic test to assess whether symptoms are related to the cyst	- High recurrence rate
US-guided aspiration with sclerotherapy	- Relatively non-invasive - Complications are rare - Effective - Possible in poor surgical candidates	- Less effective for uncooperative patients - Can not be performed if cyst communicates with biliary tree
Laparoscopic unroofing	- Technically feasible and effective in > 80% cases - Improved results with extensive fenestration and argon beam coagulation or electrocoagulation - Low recurrence rate (0%-20%) - Visualization of cyst interior (exclude other diagnoses)	- More invasive - Morbidity in up to 25% - Less effective for cysts which are superior, posterior, or deep within hepatic parenchyma - Less effective if prior surgery has been attempted
Laparotomy (resection, fenestration, or excision)	- Effective - Allows treatment of laparoscopically inaccessible cysts - Useful for cysts with complications - May perform cystojejunostomy at time of laparotomy for cysts with biliary communication	- Most invasive - Larger scars - Longer hospital stays compare to laparoscopy - Significant post-surgical morbidity

coagulation or electrocoagulation may improve the durability of results[57-61]. Recurrence has ranged from 0% to 20% with morbidity in 0% to 25%[57-60,62]. In one series, laparoscopic fenestration of simple hepatic cysts was found to be technically feasible in 90% with symptomatic relief in 95% during 38.5 mo follow-up[61]. Careful selection of patients who have not previously undergone surgical treatment and have large, symptomatic, superficial, and anterior cysts may improve outcomes[63]. US- or CT-guided cyst aspiration can be performed prior to laparoscopic fenestration in selected cases to assess whether symptoms are truly referable to the cyst[63]. Reported complications associated with laparoscopic deroofing include wound infection, bile leak, chest infection, subphrenic hematoma, and prolonged post-procedure drainage[37]. In selected cases, an open surgical procedure (fenestration, excision, or resection) may be preferred, despite longer recovery times and larger surgical scar, because of cyst location, surgeon expertise, or the presence of complicating factors[8,39,61,63,64].

Laparoscopic deroofing and cyst aspiration, followed by sclerosis, are both reasonable approaches for the majority of symptomatic simple liver cysts (Table 4)[64]. Whereas sclerosis may be less invasive and associated with lower rates of complications, laparoscopy is effective and provides the opportunity to directly examine the cyst interior to rule out etiologies other than a simple hepatic cyst[64,65].

It is important to rule out biliary communication before sclerosing a cyst, as irreversible sclerosing cholangitis has been reported as a complication[7,16,46,52,66]. The presence of cystobiliary communication may be established by endoscopic retrograde cholangiography or aspiration of bile-stained cystic fluid and is often treated by open cystojejunostomy[39], although the cystobiliary communication may be closed laparoscopically[67].

In summary, we present the case of a large, symptomatic simple hepatic cyst. We established that the cyst was the cause of symptoms only after systematically excluding alternative diagnoses through testing and empiric therapy. The usual standard of care for patients with large, simple hepatic cysts is observation, but our case demonstrates that large cysts can occasionally be responsible for symptoms. In selected cases, symptoms may respond to cyst treatment. Our treatment approach was ultrasound-guided aspiration and ethanol sclerotherapy. We believe that aspiration alone is associated with unacceptably high rates of recurrence. In our patient, excellent symptomatic and radiologic responses were achieved. In the case of cyst recurrence laparoscopic deroofing may be considered, though complications may occur in up to 25% of cases.

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CASE REPORT

Autoimmune pancreatitis metachronously associated with retroperitoneal fibrosis with IgG4-positive plasma cell infiltration

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Abstract

Retroperitoneal fibrosis is an uncommon disorder characterized by the formation of a dense plaque of fibrous tissue in the retroperitoneum, and its etiology remains unknown. Autoimmune pancreatitis is a rare type of chronic pancreatitis characterized by fibrosis with abundant infiltration of IgG4-positive plasma cells and lymphocytes and obliterative phlebitis in the pancreas. We present a case of autoimmune pancreatitis that developed 10 mo after the occurrence of retroperitoneal fibrosis. Histological findings of the resected retroperitoneal mass were marked periureteral fibrosis with abundant infiltration of IgG4-positive plasma cells and lymphocytes and obliterative phlebitis. These findings suggest a common pathophysiological mechanism for retroperitoneal fibrosis and autoimmune pancreatitis in this case. Some cases of retroperitoneal fibrosis might be a retroperitoneal lesion of IgG4-related sclerosing disease.

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Key words: Autoimmune pancreatitis; Retroperitoneal fibrosis; IgG4

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INTRODUCTION

Retroperitoneal fibrosis is an uncommon disorder characterized by the formation of a dense plaque of fibrous tissue in the retroperitoneum. Although retroperitoneal fibrosis leads to ureteral encasement and consequently hydronephrosis in most cases, it may also involve other structures in the retroperitoneum as well as in multiple unattached sites. Retroperitoneal fibrosis may be part of a systemic fibrosing disease[1,2]. Although the exact etiology of retroperitoneal fibrosis remains unknown, some cases respond well to steroid therapy[2,3].

Autoimmune pancreatitis is a recently noted clinical entity and a rare group of pancreatitis in which autoimmunity is suspected as a pathogenic mechanism. It is characterized by enlargement of the pancreas, irregular narrowing of the main pancreatic duct, elevated serum IgG4 concentrations, favorable response to steroid therapy and lymphoplasmacytic infiltration with fibrosis in the pancreas[4,5]. Recent studies have revealed occasional coexistence of autoimmune pancreatitis with other systemic exocrinopathy, such as sclerosing cholangitis[6] or sclerosing sialadenitis[7]. We have previously reported 2 other cases of autoimmune pancreatitis associated with retroperitoneal fibrosis[8]. Here, we report a case of autoimmune pancreatitis that occurred 10 mo after retroperitoneal fibrosis. We also focus on the common histopathological features with abundant IgG4-positive plasma cell infiltration in both the pancreas and retroperitoneal mass.

CASE REPORT

A 75-year-old male was admitted to another hospital with a complaint of back pain. Abdominal computed tomography (CT) showed left hydronephrosis (Figure 1A) caused by a retroperitoneal mass with encasement of the ureter (Figure 1B), which was highly suggestive of a ureteral tumor. Resection of the left lower ureter and ureterostomy were performed. Histological examination of the retroperitoneal mass revealed marked periureteral fibrosis with dense perivascular infiltration of lymphocytes and plasma cells (Figure 2A) and obliterative phlebitis (Figure 2B). These findings were compatible with retroperitoneal fibrosis. Ten months later, jaundice with abdominal pain appeared, and he was referred to our hospital for further examination.

Laboratory studies on admission were as follows: total serum bilirubin 10.4 mg/dL (normal range: 0.2-1.1 mg/dL), alkaline phosphatase 1418 IU/L (115-359 IU/L), γ-glutamyl trasnpeptidase 558 IU/L (9-70 IU/L), glutamic-

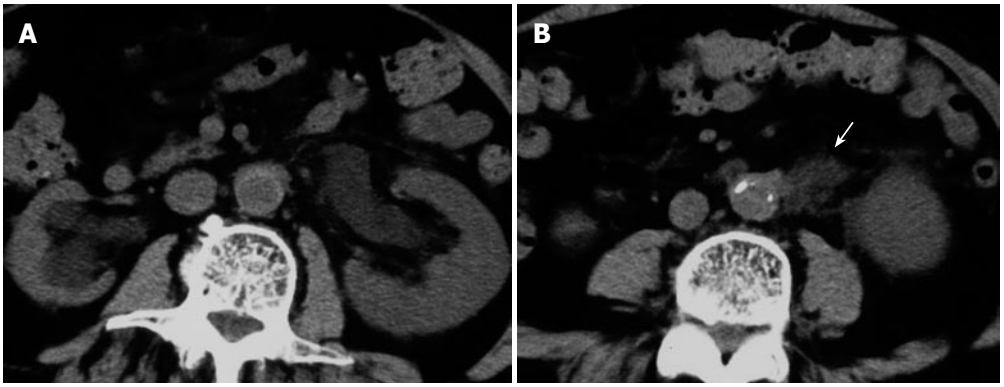

Figure 1 CT performed performed in another hospital showing left hydronephrosis (**A**) caused by a retroperitoneal mass (arrow) (**B**).

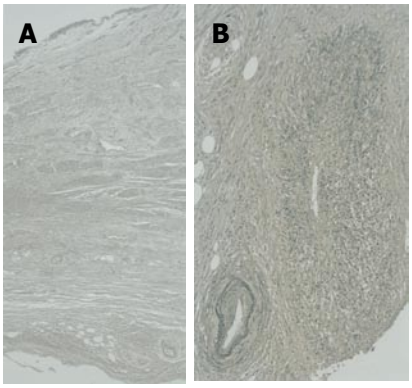

Figure 2 Histological findings of the retroperitoneal mass showing marked periureteral fibrosis with dense perivascular infiltration of lymphocytes and plasma cells (**A**, H&E) and obliterative phlebitis (**B**, Elastica Van Gienson stain).

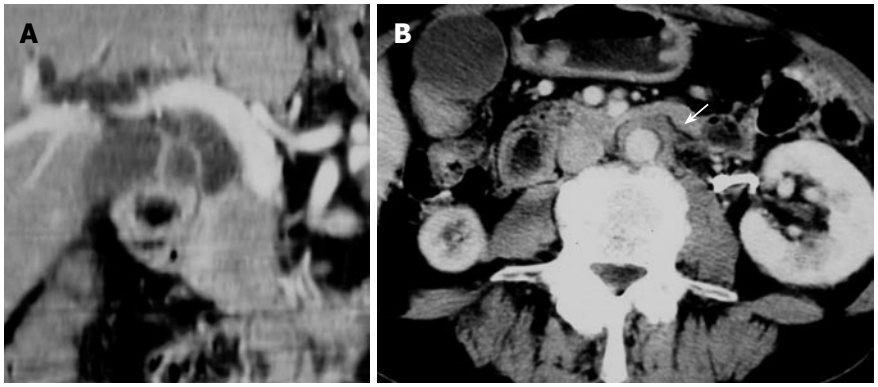

Figure 3 CT performed in our hospital showing the lower common bile duct obstructed with focally swollen pancreatic head (**A**) and soft tissue surrounding the aorta (arrow) (**B**).

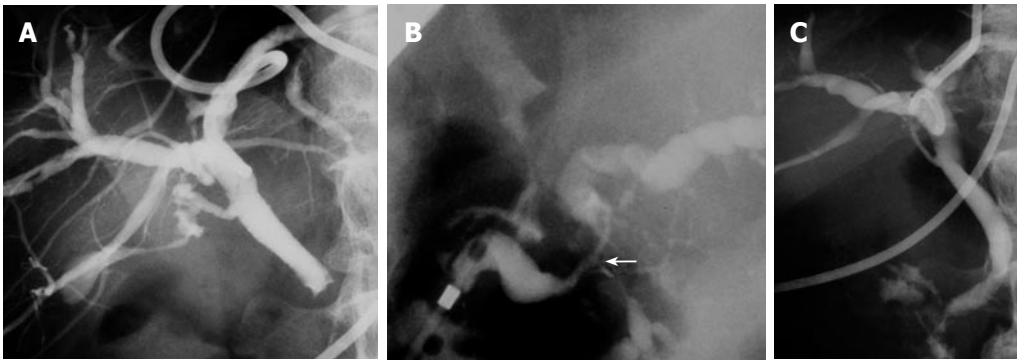

Figure 4 Percutaneous transhepatic cholangiography showing complete obstruction of the lower common bile duct (**A**) and resolution of the bile duct after steroid therapy (**C**). Endoscopic retrograde cholangiopancreatography demonstrating segmental narrowing of the main pancreatic duct in the pancreatic head (arrow) (**B**).

oxaloacetic transaminase 277 IU/L (12-32 IU/L), amylase 79 IU/L (45-155 IU/L), and elastase-1 1313 ng/mL (72-432 ng/mL). Tumor marker levels were within normal range. Although serum IgG level was 1380 mg/dL (870-1 700 mg/dL), serum IgG4 levels was elevated to 240 mg/dL (< 70 mg/dL). Antinuclear antibody and rheumatoid factor were negative. CT showed the lower common bile duct obstructed with focally swollen pancreatic head (Figure 3A) and soft tissue surrounding the aorta (Figure 3B). Magnetic resonance cholangiopancreatography (MRCP) showed the stricture of the lower common bile duct with dilatation of the upper common bile duct, but the pancreatic duct was not visualized. Percutaneous transhepatic biliary drainage was performed, and the common bile duct was completely obstructed in the lower portion (Figure 4A). Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated segmental narrowing of the main pancreatic duct in the pancreatic head (Figure 4B). Biopsy specimen taken from the major duodenal papilla during duodenoscopy revealed severe lymphoplasmacytic infiltration. Immunohistochemical studies using anti-IgG4 antibody (Binding Site, Birmingham, UK) with avidin-biotin-peroxidase complex (ABC) revealed abundant infiltration of IgG4-positive plasma cells in tissue specimens of both resected retroperitoneal mass (Figure 5A) and biopsied major duodenal papilla (Figure 5B). We diagnosed autoimmune pancreatitis and treated him with 30 mg predonisolone daily for 2 wk. The dose was

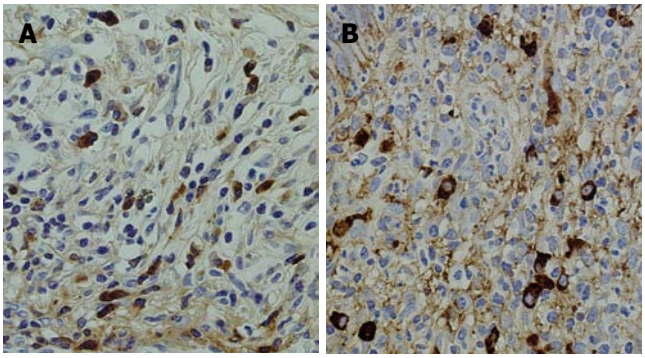

Figure 5 IgG4 immunostaining showing abundant infiltration of IgG4-positive plasma cells in tissue specimens of resected retroperitoneal mass (**A**) and biopsied major duodenal papilla (**B**).

tapered by 2.5-5 mg every 1-2 wk until a daily maintenance dose of 5 mg. After steroid therapy, findings on cholangiopancreatography and CT improved (Figure 4C), and the biliary drainage tube could be withdrawn. Serum IgG4 lowered to 83 mg/dL.

DISCUSSION

We have previously reported 2 other cases of autoimmune pancreatitis associated with retroperitoneal fibrosis[8]. A total of 9 cases including the present case have been reported[8-12]. All were middle-aged or elderly males. Autoimmune pancreatitis was synchronous to retroperitoneal fibrosis in 4 cases, and metachronous to retroperitoneal fibrosis in 3 cases. Retroperitoneal fibrosis was metachronous to autoimmune pancreatitis in 2 cases. Steroid therapy was effective for both pancreatic and retroperitoneal lesions.

Histological findings characteristic to autoimmune pancreatitis are dense lymphoplasmacytic infiltration with fibrosis and obliterative phlebitis in the pancreas[3,4]. We have reported that this lymphoplasmacytic infiltration with fibrosis is observed in the peripancreatic retroperitoneal tissue, biliary tract, and salivary glands, as well as in the pancreas of patients with autoimmune pancreatitis. Furthermore, an abundant infiltration of IgG4-positive plasma cells was observed in the various organs of patients with autoimmune pancreatitis, including the peripancreatic retroperitoneal tissue, biliary tract, salivary glands, gastric mucosa, lymph nodes, and pancreas, but an abundant infiltration of IgG4-positive plasma cells was not detected in the organs of patients with other diseases[13,14]. Major duodenal papilla also showed fibrosis with abundant infiltration of IgG4-positive plasma cells[15]. We proposed the existence of a novel clinicopathological entity, an IgG4-related systemic disease characterized by a preponderance for elderly male, and extensive IgG4-positive plasma cell and T lymphocyte infiltration of organs[14]. We also suggest that autoimmune pancreatitis is not simply a pancreatitis but that, in fact, it is a pancreatic lesion reflecting an IgG4-related systemic disease, and that in some cases only organs other than the pancreas might be clinically involved[14].

In the present case, the retroperitoneal mass, that occurred 10 mo prior to autoimmune pancreatitis, histologically showed marked periureteral fibrosis with abundant infiltration of IgG4-positive plasma cells and lymphocytes and obliterative phlebitis, which were similar findings to those in the pancreas and peripancreatic retroperitoneal tissues of patients with autoimmune pancreatitis. These findings suggest a common pathophysiological mechanism for retroperitoneal fibrosis and autoimmune pancreatitis in this case. Although the exact etiology of retroperitoneal fibrosis remains unknown, some cases might be a retroperitoneal lesion of IgG4-related sclerosing disease.

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CASE REPORT

Gastric signet-ring adenocarcinoma presenting with breast metastasis

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Abstract

Breast metastases from gastric cancer are extremely rare. A case report of a 37-year-old female with breast inflammatory invasion and ascites is described. Breast biopsy revealed carcinomatous invasion of the lymphatics from adenocarcinoma cells with signet-ring features. Estrogen (ER) and progesterone receptors (PR) and c-erb-B2 were negative. Upper gastrointestinal endoscopy revealed a prepyloric ulcerative mass. Histopathologic examination of the lesion showed infiltration from a high-grade adenocarcinoma, identical with that of the breast. Immunostaining was positive for cytokeratins CK-7 and CK-20 and CEA and negative for ER and PR. Ascitic fluid cytology was positive for adenocarcinoma cells. Mammography was not diagnostic. Abdominal CT scanning revealed large ovarian masses suggestive of metastases (Krukenberg's tumor). A cisplatin-based regimen was given but no objective response was observed. The patient died six months after initial diagnosis. A review of the literature is performed.

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Key words: Gastric adenocarcinoma; Signet-ring; Breast metastasis; Ovarian metastasis

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INTRODUCTION

Breast metastases from extra-mammary neoplasms are extremely rare. The incidence ranges between 1%-2% in clinical and 1.7%-6.6% in autopsy series[1-2]. Less than 500 cases of secondary breast tumors have been reported so far in the English speaking literature[3]. The most common primary malignancies metastasizing to the breast are malignant melanoma, lung cancer, carcinoid tumors, ovarian cancer, renal cancer, gastrointestinal carcinomas and a variety of other primaries[2-4]. Herein, a case of a woman with gastric signet-ring adenocarcinoma presenting with breast metastasis is described.

CASE REPORT

A 37-year-old female presented to our department in September 2003 with abdominal distention and painful and edematous left breast. Her medical history was unremarkable except for three attempts of *in vitro* fertilization during the last two years. Physical examination revealed ascites and marked left breast inflammation. Neither discrete tumor nodules in the breast nor any enlarged axillary nor supraclavicular lymph nodes were palpable. Mammography showed diffuse increased density and skin thickening, without evidence of mass or microcalcifications. Abdominal ultrasonography established the presence of bilateral cystic adnexal masses with thickened walls and ascitic fluid. Abdominal CT confirmed the presence of large ovarian masses suggestive of metastases (Krukenberg's tumor); however, there was no evidence of lung, liver or bone metastases. Serum tumor markers CEA, CA 15-3 and CA 19-9 were within normal limits and CA 125 was slightly elevated (63.6 U/L). Ascitic fluid cytology was found positive for adenocarcinoma. Core needle biopsy of the left breast revealed diffuse infiltration of the lymphatics in form of tumor emboli from neoplastic cells with signet-ring features (Figure 1). Immunohistochemical studies for estrogen receptor (ER) and progesterone receptor (PR) as well as for c-erb-B2 were negative. Upper gastrointestinal endoscopy demonstrated a large prepyloric ulcerative mass. Biopsy of the lesion revealed infiltration from a diffuse-type high-grade gastric adenocarcinoma with signet-ring features (Figure 2 and Figure 3). Malignant cells were positive for cytokeratins 7 and 20 (CK7, CK20) and CEA, and negative for ER and PR. Subsequent study of the breast biopsy revealed similar findings (positive staining for CK7, CK20 and CEA). The patient was treated with cisplatin-based chemotherapy; but she showed no objective response and died six months after the initial diagnosis.

DISCUSSION

Metastatic breast involvement from extra-mammary

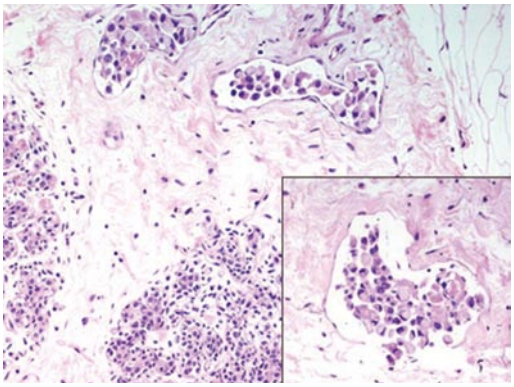

Figure 1 Breast biopsy showing breast lymphatic invasion from neoplastic cells with signet-ring features (Hematoxylin & eosin, 200 ×, 400 ×).

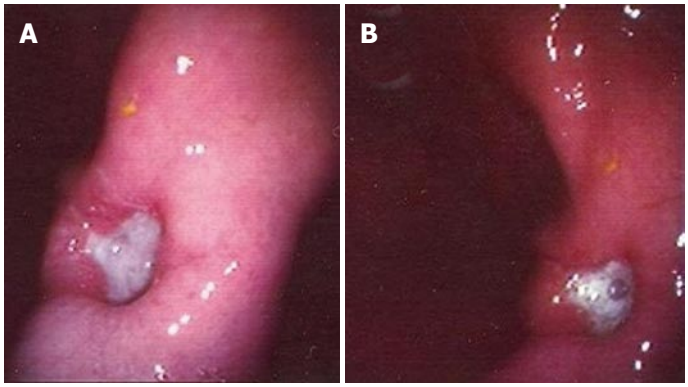

Figure 2 (**A** & **B**) Upper GI endoscopy of the patient, showing a large ulcerative lesion in the prepyloric region.

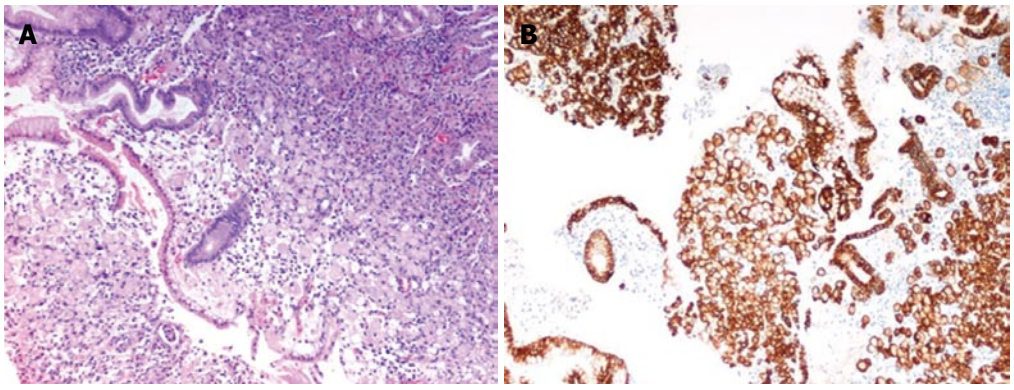

Figure 3 Gastric biopsy showing infiltration from neoplastic cells with signet-ring features (Hematoxylin & eosin, 100 ×). Right picture shows immunohistochemical stain for CK-7 (Hematoxylin & eosin, 100 ×).

primaries is unusual. Most cases are related with extensive disease burden and carry an unfavorable prognosis[5]. Breast invasion is the initial manifestation of the disease in 25%-40% of the cases[2]. Mammographically, metastatic tumors in the breast present as well-circumscribed nodules without microcalcifications, mimicking benign breast lesions[5]. Calcifications may be seen in metastatic deposits from ovarian carcinoma with psammoma bodies[6]. The most common localization of metastatic lesions is the upper-outer quadrant of the left breast. Breast involvement is bilateral in 25% of the cases, and there is concomitant axillary lymph node enlargement in up to 15%[5]. The occurrence of multiple tumor nodules is unusual and diffuse involvement mimicking inflammatory breast carcinoma is rare[7,8]. The latter clinical manifestation, due to lymphatic invasion, is rapidly progressive, and may produce clinical signs of breast involvement before a firm metastatic nodule is formed, which is even more common in hematological malignancies (leukemias, lymphomas).

Breast metastases from gastric cancer are extremely rare. To date, only 25 cases have been reported in the English speaking literature and are mentioned in Table 1. Signet-ring features have been reported in 13 cases. From reports with available data, it seems that breast involvement usually follows the detection of gastric carcinoma. Breast metastasis as initial manifestation of a primary gastric tumor, as in our case, has been reported in 10 cases. In half of these cases breast involvement had the clinical appearance of inflammatory breast cancer.

Breast metastases from gastric carcinoma differ from primary breast cancer in their histopathologic characteristics. Immunostaining is usually negative for estrogen and progesterone receptors[9,10] as well as for c-erbB-2[3]. GCDFP-15 (gross cystic disease fluid protein-15) is also absent[10]. There is no evidence of an *in situ* component[3,5] and a lack of desmoplastic response[3]. On the contrary, lymphatic tumor emboli and epithelial markers like CK7, CK20 and CEA are usually present[3,5].

In our case, the presence of signet-ring cells within breast lymphatics, the absence of mass or microcalcifications in the mammography, the lack of an *in situ* component and hormone receptor negativity strongly suggested an extra-mammary primary. It is of noted interest that Steinbrecher and Silverberg in 1976[11] first described primary signet-ring carcinoma of the breast, as a subtype of lobular carcinoma, with aggressive biological behavior and an increased tendency to metastasize in the abdomen and in the gastrointestinal tract compared to other histological subtypes[9,10]. Signet-ring carcinoma of the breast metastatic to the stomach has also been reported[12,13].

The presence of cystic ovarian masses suggested metastatic ovarian involvement (Krukenberg's tumor). Unfortunately, the patient's unfavorable clinical condition did not allow histological confirmation. Metastatic involvement of the breast and the ovaries is extremely rare and has been reported in only 5 cases so far. Selective invasion of hormone-dependent organs seems quite intriguing, especially in pre-menopausal women. Increased blood supply of the breast has been proposed as the

Table 1 Patients' characteristics[1] in reported cases of breast metastasis from gastric cancer

n	Reference[2]	Age	Histology	Presentation after initial diagnosis (mo)	Clinical presentation	Localization	Other metastatic sites
1	Reitmann 1908	33	Scirhous	-	-	R+L[3]	-
2	Kreibich 1909	65	Scirhous	-	-	R	Skin
3	Mourier 1910	31	Mucinous	-	-	L	Liver, pancreas
4	Stahr 1922	46	Anaplastic	-	-	R+L	-
5	Dawson 1936	25	Mucinous	-	-	R+L	Ovaries
6	Abrams 1949	-	-	-	-	-	-
7	Sandison 1959	56	Signet-ring cell	-	-	L	-
8	Hajdu 1972	-	Adenocarcinoma	-	-	L	-
9	Silverman 1974	-	Mucin-producing	Concomitant[4]	-	-	-
10	Toombs 1977	-	-	-	-	-	-
11	Satake 1980	39	Signet-ring cell	Concomitant	Nodule	L	-
12	Togo 1980	70	Signet-ring cell	Concomitant	Nodule	L	-
13	Nielsen 1981	59	Mucinous	Concomitant	Nodule	L	-
14	Champault 1982	65	Adenocarcinoma	Concomitant	Nodule	L	-
15	Kasuga 1986	48	Signet-ring cell	31	Nodules	R+L	-
16	Alexander 1989	28	Mucinous differentiation	Concomitant	Nodules	R+L	Lymph nodes
17	Hamby 1991	31	Signet-ring cell	Concomitant	Nodule	R	Lymph nodes, ovaries
18	Cavazzini 1993	-	Signet-ring cell	-	Inflammatory	-	-
19	Domanski 1996	48	Signet-ring cell	Concomitant	Nodule	L upper-outer	L supraclavicular
20	de la Cruz Mera 1998	61	Signet-ring cell	13	Nodule	L upper-outer	Pleura
21	Briest 1999	46	Signet-ring cell	2	Inflammatory	R+L	Bilateral axillary nodes
22	Kwak 2000	41	Signet-ring cell	Concomitant	Inflammatory	L	Ovaries[5]
23	Kwak 2000	23	Signet-ring cell	Concomitant	Inflammatory	R	Axillary nodes
24	Madan 2002	39	Signet-ring cell	3	Nodule	R	Ovaries, peritoneum
25	Di Cosimo 2003	39	Signet-ring cell	1	Nodules	R+L	Ovaries, peritoneum, skin
26	Boutis 2005	37	Signet-ring cell	Concomitant	Inflammatory	L	Ovaries[5], ascites

[1] Clinical information is mentioned in the table only if available by the authors.
[2] References Reitmann 1908, Kreibich 1909, Mourier 1910, Stahr 1922, Dawson 1936, Satake 1980, Togo 1980 are included in Hamby 1991[2].
[3] R: right; L: left.
[4] Breast metastasis was the initial manifestation of disease.
[5] No histological confirmation.

mechanism for the increased incidence of breast metastasis in pre-menopausal women[14-15]. On the other hand, gastric cancer seems to have a more aggressive biologic behavior in younger age groups, where hormonal factors are implicated[16]. The appearance of breast metastases in men with gynecomastia supports the latter hypothesis[17]. In our patient, hormonal therapy for the induction of ovulation may have influenced the natural history of the disease[1]. However, no conclusions about the pathogenesis of the phenomenon can be drawn.

Metastatic disease of the breast is rare, and usually has atypical clinical and mammographic presentation. In cases of breast inflammation or lumps, biopsy should be performed, even in the presence of an extra-mammary neoplasm. Likewise, peculiar histological features from malignant breast lesions should be co-estimated with other clinical parameters toward the diagnosis of metastatic disease. Thus, avoidance of unnecessary radical surgical procedures and an adequate treatment is more probable to be given in these patients.

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CASE REPORT

Multiple pyogenic liver abscess

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Abstract

Multiple pyogenic liver abscesses have been rarely described. We report a fatal case of multiple pyogenic liver abscesses affecting a 38-year-old woman requiring surgical drainage. Evolution was marked by occurrence of a septic shock with multi-organ system failure. The patient died 48 h after surgery. Causes, therapeutics and outcome of the disease are discussed.

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Key words: Liver abscess; Septic shock; Outcome

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INTRODUCTION

The mortality rate of pyogenic liver abscess is 11%-31%[1]. The overall mortality is high in patients with multiple liver abscesses[2]. However, multiple pyogenic liver abscesses are not frequently reported in the literature. We report a fatal case of multiple and gigantic liver abscess affecting a 38-year-old woman.

CASE REPORT

A 38-year-old woman was admitted to our hospital for abdominal pain in the right upper quadrant, fever, vomiting and anorexia as well as chills and jaundice. On examination she had marked tenderness in the right upper quadrant. The temperature was 40.1 ℃, the pulse was 119, and the respiratory rate was 30/min. The blood pressure was 80/30 mmHg. The patient did not drink alcohol.

Laboratory tests performed on admission revealed 41.5% hematocrit, 26 300 white-cell count (per mm^3), 62 000 platelet count (per mm^3), 30% prothrombin time, 269 μmol/L total bilirubin, 170 μmol/L conjugated bilirubin, 11 mmol/L urea nitrogen, 42 μmol/L creatinine, 4.5 mmol/L glucose, 117 mmol/L sodium, 4 mmol/L potassium, 87mmol/L chloride, 16 mmol/L carbon dioxide, 51U/L aspartate aminotransferase, 21 U/L alanine aminotransferase and 208 U/L creatine kinase.

Ultrasonography of the abdomen revealed that the hepatic parenchyma appeared diffusely heterogeneous, with hypoechoic foci in the right lobe, suggesting the presence of abscess or multiple hydatid cysts.

CT scan of the abdomen (Figures 1A-B) before and after intravenous injection of contrast material confirmed the presence of multiple irregular abscesses varying in size, predominantly in the right lobe of the liver.

Microbiological culture was performed (hemoculture) and the patient received intravenously cefotaxime, gentamicin, metronidazole, omeprazole, fluid, and electrolytes. Surgical drainage was performed 48 h after hospital admission. *Escherichia coli, Bacteroides fragilis and prevotella oralis* were found in cultures of the pus.

Evolution was marked by occurrence of septic shock with multi-organ system failure. The patient died 48 h after surgery.

DISCUSSION

Pyogenic liver abscesses are found in 0.3%-1.4% of autopsies[3]. Patients with diabetes mellitus, immune deficiency, sickle cell anemia, malignancy, and liver transplants are at a greater risk for developing liver abscess[1]. The majority of pyogenic liver abscesses are caused by infection originating in the biliary or intestinal tracts[4]. About 10% of pyogenic liver abscesses develop as a result of bacteria entering the liver via the hepatic artery[5]. In the majority of cases, more than one organism has been isolated from their abscesses[6]. The most commonly infecting organisms are Gram-negative aerobes and *Escherichia coli* is the most frequently encountered[7]. In our case three organisms were identified, suggesting that pyogenic liver abscesses are caused by infection originating in the intestinal tract. Without appropriate diagnosis and treatment, pyogenic liver abscesses are almost uniformly fatal[2,4]. Early diagnosis as well as treatment with appropriate antibiotics and selective drainage can substantially reduce mortality. Our observation has confirmed this hypothesis.

Antibiotic therapy should include penicillin, which is

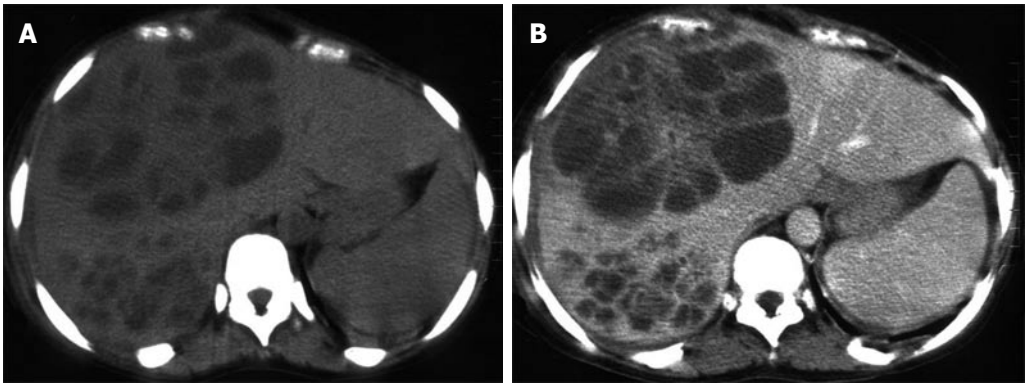

Figure 1 CT scan without (**A**) and with (**B**) contrast injection of the abdomen showing multiple irregular liver abscesses in the right hepatic lobe.

effective against *E coli*, *K pneumonia*, *bacteroides*, *enterococcus*, and *anaerobic streptococci*[1]. Antibiotics alone are effective for only a few patients and most patients require percutaneous aspiration or catheter drainage guided by ultrasonography or computed tomography[1]. In our case open surgery was performed because the patient suffered from multiple liver abscesses and septic shock.

The overall mortality may be as high as 30%-40% in patients with multiple liver abscesses, malignant biliary obstruction, inadequate drainage, and immunodeficiency[1,2]. In our case no pathological antecedent was found, however we cannot exclude immunodeficiency in this woman.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

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24-25 March 2006
Sydney - NSW
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10th International Congress of Obesity
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Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
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Prague hepatology 2006
14-16 September 2006
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22 July 2006-1 August 2006
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6th Annual Gastroenterology And Hepatology
15-18 March 2006
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c.chase@imedex.com

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Los Angeles
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Annual Postgraduate Course
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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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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Statistical data

Present as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as γ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2 not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further mention.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyr*A, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H pylori, E coli, etc.*

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